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DESTRUCTION OF TETANUS ANTITOXIN BY CHEMICAL AGENTS

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OBJECT AND PLAN OF WORK

The ultimate object of the work herein described is a solution of the problem of the chemical nature of antitoxins and their preparation in the pure state. That this would be attained was not expected, in view of the numerous previous investigations which left these problems unsolved. But it seemed highly probable that data would be obtained which would throw some light on the subject and serve as guides for other investigations.

Up to the present time numerous investigators have attempted to separate antitoxins from their associated proteins, but without complete success. The well-known tetanus and diphtheria antitoxins are examples of preparations containing all or nearly all of the immunity units present in the original serums, but only a part of the proteins. Thus, Homer (8)[1] concentrated a tetanus serum containing 100 units per cubic centimeter and 6 per cent of protein, obtaining a product that contained 900 units per cubic centimeter and 19 per cent of protein. In this process 10 per cent of the antitoxic units were lost, the final product was 9 times as potent as the original serum and contained but 3 times as much protein. The failure of all attempts to obtain a protein-free antitoxin preparation has led some investigators to the conclusion that the antibody (or group of antibodies) which constitutes the antitoxin is one of the serum proteins, and hence can not be completely separated from protein. The concentration of antitoxin without a similar concentration of protein is regarded by others as an indication that the antitoxin may be a body of non-protein nature.

Under these conditions any test which would conclusively decide whether an antitoxin is or is not identical with a serum protein would have both a practical and a theoretical interest. Accordingly, the fol-

[1] Reference is made by number (italic) to "Literature cited," pp. 494–495.

lowing test was decided upon because of its promising nature. If an antitoxin—for example, tetanus antitoxin—is a substance of nonprotein nature, it should be possible to prepare artificial digestion mixtures containing the antitoxic serum or derived globulin in such a manner that the protein would undergo digestion without loss of antitoxin. Appropriate chemical measurements would indicate the extent to which proteolysis has taken place, while inoculation experiments on guinea pigs would indicate whether there was any loss of antitoxic units. If, on the other hand, the antitoxin is a protein, and its power to immunologically neutralize the corresponding toxin is a function of the intact protein molecule, then the antitoxin would be destroyed in every case where the proteins had undergone cleavage, regardless of whether the cleavage was caused by a proteolytic enzym or other chemical agent. Due regard must, of course, be had for the possible destruction of the toxin by the chemical agents used.

However, these theoretical considerations were not the only ones that prompted the present investigation. For practical reasons numerous investigators studied the possibility of immunizing animals by administering the antitoxin by mouth. Some found that tetanus and diphtheria antitoxins were destroyed in the digestive tract; others found that the animals could be so immunized. The present work on the effect of digesting tetanus serum or derived globulin *in vitro* was expected to throw some light on the fate of immunity units administered *per os*.

WORK OF PREVIOUS INVESTIGATORS

Table I briefly summarizes the conclusions of the more important investigations on this subject. Of the two on tetanus serum by Carrière (*4*) and McClintock and King (*9*), it is doubtful whether either led to entirely correct conclusions. McClintock and King state (*p. 702*)—

> . . . we are now able to take series after series of guinea-pigs or rabbits, and by the oral administration of [tetanus, diphtheria] antitoxin save 100 per cent of the treated animals, when the antitoxin is administered before the toxin; while the untreated ones, receiving the same dose of poison, invariably die . . .

In their experiments with diphtheria and tetanus antitoxins (*p. 713*) administered to men by mouth they demonstrated the presence of the antitoxin in the blood of the men who had swallowed it a few days before the guinea-pig inoculation test. Their numerous positive immunizations by mouth led them to conclude that—

> . . . the diphtheria or tetanus antitoxin, which had been administered to the individual, had resisted digestion and had been absorbed in sufficient quantities to cause the protective antitoxin to be present in the blood of the individual . . .

These remarkable results by McClintock and King, although apparently obtained under carefully controlled conditions, have not carried conviction with them; they have not served as a starting point for

other investigations. Subsequent confirmation or contradiction of their work was not found, but practically all workers before them found that immunization by administration *per os* was not practicable. The immune bodies were destroyed or neutralized; most probably they were destroyed by the acid, the alkali, and the proteolytic enzyms of the digestive tract acting singly or in combination.

TABLE I.—*Summary of results of previous investigations on the action of proteolytic enzyms on antitoxins*

Investigation.	Digestive tract.	Pepsin-hydrochloric acid.	Hydrochloric acid.	Trypsin.
Belfanti and Carbone (*2*) diphtheria serum and diphtheria antitoxin.	.....	Destroyed.....	Destroyed by 0.1 per cent hydrochloric acid in a few hours.	Destroyed.
Carrière (*4*) tetanus serum	.....	Not affected in 24 hours.	.....	Destroyed appreciably in 24 hours. Alkali absent.
Dzierzgowski (*5*) diphtheria serum.	.....	Destroyed almost completely in 10 hours.	Destroyed almost completely by 0.5 per cent hydrochloric acid in 24 hours.	Not destroyed by pancreatic juice in 12 hours.
Nicolas and Arloing (*11*) diphtheria serum.	Destroyed.....	.....	.....	.....
Pick (*12*) diphtheria serum.	.....	.....	.....	Destroyed two-thirds by trypsin-alkali in 9 days.
McClintock and King (*9*) diphtheria serum and tetanus serum.	Not destroyed...	.....	.....	.....
Mellanby (*10*) diphtheria serum.	.....	Destroyed.....	Not affected by 0.25 per cent hydrochloric acid (time not stated).	Destroyed slowly in 9 days.

If it be assumed that McClintock and King actually immunized animals and man by *per os* administration and detected the antitoxin in the blood of individuals who had swallowed antitoxin, it does not follow that the antitoxin resisted digestion. It may have been absorbed from the digestive tract before the antitoxin had been destroyed.

Carrière (*4*) found that tetanus serum in pepsin-hydrochloric acid lost none of its immunizing power in 24 hours, a finding that is undoubtedly erroneous. He correctly found that trypsin appreciably destroyed the antitoxic properties in 24 hours.

On account of the many properties common to both diphtheria and tetanus serums, some of the results on diphtheria were included in Table I because they undoubtedly throw light upon one another. This table speaks for itself. It shows that in general the antibodies present in tetanus and diphtheria serums, or in globulin concentrates obtained from them, do not resist the action of the chemical agents present in the digestive tract. There are slight inconsistencies, but these do not invalidate the above generalization. Thus, Dzierzgowski (*5*) in 1899 concluded that diphtheria serum does not lose any of its immunizing power when mixed for 12 hours with active pancreatic juice (*p. 350*). The recently discovered antitrypsin (*7, p. 121*) present in normal and patho-

logical blood should have indicated to Dzierzgowski that this was too short a digestion period, for only three years later Pick (*12, p. 386*), using but 10 guinea pigs in his digestion experiments, correctly found that two-thirds of the antitoxin was destroyed by trypsin if given time enough—9 days. Mellanby's statement (*10, p. 405*) that 0.2 per cent hydrochloric acid has no destructive action on diphtheria antitoxin is at variance with that of numerous workers who found that it was destroyed.

On the practical side the net results of the numerous investigators, a few of whom are mentioned in Table I, indicate the impracticability of administering antitoxins by mouth. On the theoretical side they throw very little light on the problem of the nature of the antibodies themselves. The various investigators who attempted to separate a pure antitoxin from associated protein naturally sought a method by which the pure antitoxin would be obtained free or nearly free from protein, regardless of whether the proteins were destroyed during the purification. The converse did not occur to any of the workers whose publications have been studied—that is, to separate the antitoxin from the associated proteins, leaving the proteins entirely intact, but destroying the antitoxin.

For the purpose of proving that the antitoxin is not of protein nature, it is obviously immaterial whether one obtains pure antitoxin free from protein or the pure protein free from antitoxin so long as the separation actually is made. No one has succeeded in making the first separation; the writers have succeeded in making the second.

In the experiments here described the different antitoxic preparations were exposed to the action of trypsin-sodium-carbonate or to pepsin-hydrochloric-acid solutions for comparatively long periods of time, with suitable controls. (See Table III.) The extent to which digestion took place was then measured. The antitoxin remaining in the mixtures was estimated by inoculation tests on guinea pigs, carried out with slight modifications according to the methods described by Rosenau and Anderson (*13*). Anthrax serum was studied first, but as the results obtained were inconclusive, they are only briefly mentioned after the data on tetanus serum and tetanus antitoxin.

EXPERIMENTAL MATERIALS

Table II contains a brief description of the antitoxic preparations and proteolytic enzyms used.

GUINEA PIGS.—A total of 440 guinea pigs were used. These were obtained from the Bureau of Animal Industry's stock of 3,000 or more continually on hand at the Bureau's Experiment Station at Bethesda, Md. These animals have been bred for years from selected hardy stock and are undoubtedly very superior for experimental purposes. Only the obviously healthy animals, weighing between 320 and 380 gm. were selected at the station and forwarded to the Bureau's animal room 2 or

3 days before they were used for experimental purposes. Out of these 440, 250 died during the various observation periods. It is believed that without exception the deaths were all due to tetanus. No post-mortem examinations were made, it being evident that the deaths were due to tetanus either from the symptoms or from the time and number of deaths. In all cases the symptoms of tetanus were observed when recording the death as due to tetanus, except in experiment 21, in which 36 guinea pigs were inoculated between 3 and 4 p. m. None were "down" the next morning, but when next observed, at 9 p. m., 30 hours after inoculation, 23 were found dead without anyone's having observed any symptoms, while 12 controls were alive. Deaths were recorded as tetanus, as the time, grouping, etc., practically precluded other possibilities.

TABLE II.—*Preparations used in experiments*

Designation of preparation.	Where obtained.	Maker's statement of number of antitoxic units in 1 c. c. of the preparation.
Tetanus antitoxin No. 420F	Lederle antitoxin laboratories	300.
Tetanus serum No. 374	do	202.
Tetanus antitoxin X	A mixture, total volume 55 c. c. of the following makes: Slee; Parke, Davis & Co.; and Mulford.	Average 236.
Tetanus serum No. 268	Lederle antitoxin laboratories	200.
Pepsin 1	A 100-gm. sample of Parke, Davis & Co. 1:3000, purchased about May, 1912.	
Pepsin 2	A 200-gm. sample of pepsin puriss, Gruebler, imported about March, 1913.	
Trypsin 3	A 50-gm. sample of "trypsin Merck," purchased August, 1913.	
Trypsin 4	A 30-gm. sample of "trypsin Merck," purchased November, 1916.	
Tetanus toxin F4	Hygienic Laboratory, United States Public-Health Service.	Standard toxin test dose, 0.0007 gm.
Tetanus toxin F1	do	Do.

The death of a guinea pig within a few days after its receipt from the Bureau Experiment Station is such a rarity that it may be disregarded. But 1 occurred out of the 440 guinea pigs. By reason of the selection at the station such deaths are more likely to be due to mechanical injury than disease. The comments by Wahl (*14, p. 227*) on the well-known susceptibility of guinea pigs to intercurrent infections and sudden fatal epidemics, pneumonia, pleurisy, ascites, etc., while true of ordinary guinea pigs, certainly do not apply to the stock maintained by the Bureau.

PREPARATION OF DIGESTION MIXTURES.—These were prepared as indicated in Table III and used in sets of five for obvious reasons. Mixture A contained only the serum or antitoxin, and served as a standard for comparing the antitoxin contents of the different mixtures. Guinea pigs inoculated with quantities of mixture A equivalent to 0.1 unit of antitoxin or more always survived the test dose of toxin. Mixture B was to show the effect of sodium carbonate or hydrochloric acid on the antitoxin. Mixture C was to show the effect of trypsin or pepsin on the antitoxin. In mixture D the proteins present in the tetanus serum or antitoxin were digested by pepsin-hydrochloric-acid or trypsin-sodium-carbonate. In experiment 6 sodium hydroxid was used instead of sodium carbonate, with practically the same results. In mixture E the effect of the pepsin-hydrochloric-acid or trypsin-sodium-carbonate on the toxin was shown. The antitoxin and toxin were mixed one hour before inoculation; hence, it was necessary to be certain that the reagents at their final dilution under the conditions of the experiments do not destroy any of the toxin.

TABLE III.—*Composition of digestion mixtures and analytic data*

EXPERIMENTS 1 TO 3.1 (1 C. C. OF MIXTURE TO CONTAIN 60 UNITS)

Mixture.	Antitoxin or serum.		Water.	Acid or alkali.		Enzym.		Acid or alkali used to flocculate 2 c. c. of mixture.	Digestion period.	In 1 c. c. of antitoxin.	
	Number.	Quantity.		Kind.	Quantity.	Kind.	Quantity.			Total coagulable protein.	Amino nitrogen.[a]
		C. c.	*C. c.*		*Mgm.*		*Mgm.*	*C. c.*	*Days.*	*Mgm.*	*Mgm.*
A	Antitoxin 420 F	10	40. 0	Sodium carbonate	0	Trypsin 4	0	0.05 *N*/50 acetic acid	0	59. 0	0. 53
B	do	10	40. 0	do	250	do	0	.9 *N*/5 acetic acid	15	57. 7	.....
C	do	10	40. 0	do	0	do	200	.1 *N*/50 acetic acid	10	25. 7	3. 43(13)
D	do	10	40. 0	do	250	do	200	.9 *N*/5 acetic acid	13	21. 2	3. 22(14)
					250						[b] 7. 06
E	Anthrax globulin	2. 5	47. 5	do		do	200				

EXPERIMENTS 4 TO 5.1 (1 C. C. OF MIXTURE TO CONTAIN 40 UNITS)

										In 1 c. c. of serum.	
A	Serum 374	10	40	Sodium carbonate	0	Trypsin 4	0		0	.....	0. 87
								0. 7 *N*/50 acetic acid	6	74. 2	. 82
								. 7 *N*/50 acetic acid	17	75. 7	. 76
B	do	10	40	do	250	do	0	. 9 *N*/5 acetic acid	6	71. 5	. 76
								. 9 *N*/5 acetic acid	17	76. 2	. 68
C	do	10	40	do	0	do	200	. 1 *N*/50 acetic acid	6	32. 5	5. 66
								. 1 *N*/50 acetic acid	17	14. 5	6. 62
D	do	10	40	do	250	do	200	. 9 *N*/5 acetic acid	6	31. 5	3. 91
								. 9 *N*/5 acetic acid	17	16. 5	4. 29
											[b] 9. 91
E	Anthrax serum 160	10	40	do	250	do	200				

[a] Figures in parentheses are time (days) of digestion.

[b] Total amino nitrogen by hydrochloric acid hydrolysis. See p. 482.

TABLE III.—*Composition of digestion mixtures and analytic data*—Continued

EXPERIMENTS 6 AND 6.1 (1 C. C. OF MIXTURE TO CONTAIN 47 UNITS)

Mixture.	Antitoxin or serum.		Water.	Acid or alkali.		Enzym.		Acid or alkali used to flocculate 2 c. c. of mixture.	Digestion period.	In 1 c. c. of antitoxin.	
	Number.	Quantity.		Kind.	Quantity.	Kind.	Quantity.			Total coagulable protein.	Amino nitrogen.[a]
		C. c.	*C. c.*		*C. c.*		*Mgm.*	*C. c.*	*Days.*	*Mgm.*	*Mgm.*
A	Antitoxin X	10	40	Sodium-hydroxid solution *N*/172.	0	Trypsin 3		0.6 *N*/50 acetic acid	8	[b] 110.9	1.73
B	do	10		do	40	do		.1 *N*/50 acetic acid	8	25.7	11.51
C	do	18	40	do	0	do	200	.6 *N*/50 acetic acid	8	110.9	1.69
D	do	10		do	40	do	200	.1 *N*/50 acetic acid	8	25.7	11.33
											[c] 13.73
E	Anthrax serum 160	10		do	40	do	200				

EXPERIMENT 20 (1 C. C. OF MIXTURE TO CONTAIN 60 UNITS)

A	Antitoxin 420 F	10	40	2 per cent hydrochloric acid.	0	Pepsin 1		0.1 *N*/50 acetic acid	12	61.6	
									14	63.5	
B	do	10	35	do	5	do		.55 *N*/5 sodium hydroxid.	12	62.0	
									14	62.0	
C	do	10	40	do	0	do	100	.10 *N*/50 acetic acid	12	[b] 49.0	
								.00	14	57.2	
D	do	10	35	do	5	do	100	.55 *N*/5 sodium hydroxid.	12	1.5	
									14	2.3	
E	Normal horse serum	10	35	do	5	do	100				

EXPERIMENT 21 (1 C. C. OF MIXTURE TO CONTAIN 60 UNITS)

A	Antitoxin 420 F	10	40	2 per cent hydrochloric acid.	0	Pepsin 1		0.1 *N*/50 acetic acid	3	65.1	0.62(8)
B	do	10	35	do	5	do		.55 *N*/5 sodium hydroxid.	3	59.7	0.67(8)
C	do	10	40	do	0	do	100	.00	3	58.5	0.76(8)
D	do	10	35	do	5	do	100	.55 *N*/5 sodium hydroxid.	3	3.5	1.82(8)
											[c] 7.06
E	Normal horse serum	10	35	do	5	do	100				

EXPERIMENT 22 (1 C. C. OF MIXTURE TO CONTAIN 40 UNITS)

										In 1 c. c. of serum.	
A	Serum 268	10	40	2 per cent hydrochloric acid.	0	Pepsin 2		0.6 *N*/50 acetic acid	4	97.5	0.56(5)
B	do	10	35	do	5	do		.5 *N*/5 sodium hydroxid.	4	95.2	1.01(5)
C	do	10	40	do	0	do	100	.3 *N*/50 acetic acid	4	92.0	1.12(5)
D	do	10	35	do	5	do	100	.4 *N*/5 sodium hydroxid.	4	4.7	2.73(5) [c] 11.78
E	Normal horse serum	10	35	do	5	do	100				

[a] Figures in parenthesis are time (days) of digestion. [b] See p. 481. [c] Total amino nitrogen was obtained by hydrochloric-acid hydrolysis. See p. 482.

The mixtures were contained in wide-mouthed 100-c. c. bottles, and were closed with rubber stoppers tied firmly in place. The mixtures were always saturated with chloroform and kept in an incubator room at 37.5° C. Culture tests made from time to time showed that the mixtures were sterile.

ANALYTIC DATA

At suitable intervals, usually the day before or after the guinea pigs were inoculated, chemical analyses of the mixtures were made for the purpose of ascertaining the extent of protein digestion. The determinations made were coagulable protein and amino nitrogen. Inasmuch as details on the preparation and analyses of digestion mixtures have been published elsewhere (3), they will be omitted here.

TOTAL COAGULABLE PROTEIN.—The protein was precipitated, coagulated, centrifuged, dried, and weighed in centrifuge tubes (heavy glass bacteriological test tubes), the outer dimensions of which were: Length 95 mm., diameter 17 mm. Eight such tubes, cleaned, dried, and weighed to 0.1 mgm., were used at a time, two for each of the mixtures A, B, C, and D. No analyses were made of mixture E. Into each tube 7 c. c. of water and 2 c. c. of digestion mixture or 9 c. c. of water and 1 c. c. of serum or antitoxin were measured, with Ostwald pipettes of 1 and 2 c. c. delivery for the latter. Each tube was warmed over a Bunsen burner, and sufficient dilute acetic acid or sodium-hydroxid solution was added to precipitate completely the protein, after which the contents of the tube were brought almost to a boil. The tubes were then centrifuged for 20 to 25 minutes at about 2,400 revolutions per minute. When the flocculation had been properly performed, the coagulated protein packed firmly to the bottom, leaving a water-clear supernatant fluid which may be poured off without appreciable loss of precipitate, even if the tube is inverted. The tubes were then immersed in a sulphuric-acid-potassium-dichromate cleaning mixture, rinsed, and dried on the outside to make certain that there was no adherent dirt. The tubes containing the moist precipitates were dried to constant weight at room temperature in a sulphuric-acid desiccator evacuated to about 10 mm. of mercury. The drying was not difficult; usually a single drying of 24 hours was sufficient, provided the acid was renewed very frequently—that is, after two dryings. In every case the drying was continued until the loss in weight of a precipitate did not exceed a few tenths of a milligram. The difference between the weight of the tube empty and the weight with the precipitate gave the weight of coagulable protein.

These tubes, which had been numbered with hydrofluoric acid, after being used were cleaned with hot dichromate mixture, washed, dried in the hot-air oven, placed in the desiccator, and weighed, ready for the next determinations. This method for total coagulable protein gives

quick and accurate results. Thus in experiment 6 the following were obtained with 2 c. c. of the mixtures containing 0.4 c. c. of antitoxin X: Mixture A, 43.3, 44.8 mgm.; mixture C, 43.8, 44, 44.8, 45.4 mgm.; mixture B, 9.8, 10.2, 10.2, 10.5 mgm.; mixture D, 10.4, 10.5 mgm. In this experiment an error was made by the addition of trypsin to mixture B instead of C, resulting in two pairs of identical mixtures, one of which contained only the antitoxin, the other trypsin-sodium-hydroxid in addition.

The most difficult step in the determination of coagulable protein is the complete flocculation of the protein. The above-mentioned close duplicates probably will not be obtained by the inexperienced or those who do not realize the effect of small quantities of acid or alkali in this determination. This point is well illustrated by the following data obtained in experiment 20 (see Table III): To 2 c. c. of mixture C containing 0.4 c. c. of antitoxin 420F., 7 c. c. of water were added, and the protein was flocculated with 0.10 c. c. *N*/50 acetic acid. The total coagulable protein obtained was 19.4 and 20 mgm. The average of these two, calculated to 1 c. c. of antitoxin, gave 49 mgm., the result given in Table III. Two days later the determinations were repeated, but neither acid nor alkali was used for flocculation. Obtained 22.5 and 23.3 mgm., which, calculated as before, gave 57.2 mgm. total coagulable protein per 1 c. c. of antitoxin. So small a difference as 0.1 c. c. of *N*/50 acetic acid made an appreciable difference in the result—that is, 8 mgm.

It will be noticed that the figure for mixture C, 57.2 mgm., is appreciably lower than that for mixture A, 63.5 mgm., as if 9 per cent of the protein had been digested past the coagulable stage. Mixture C contained pepsin only, and this in all probability exerted no digestive action in the neutral solution. In general it was found that the addition of pepsin or trypsin to a mixture lowered the amount of protein coagulated when the coagulation was attempted immediately after the addition. Thus, in experiment 3, eight 2-c. c. portions of mixture B were pipetted into as many centrifuge tubes. Four were coagulated in the usual way with 0.9 c. c. of *N*/5 acetic acid. There were obtained 22.1, 22.2, 24.0, and 24.2 mgm. of coagulable protein. To the other four tubes weighed portions of trypsin 4 were added, varying from 27 to 72 mgm., and the coagulation carried out as before without allowing any time for the trypsin to act proteolytically. The coagulable protein obtained amounted to 19.0, 19.5, 20.2, and 20.3 mgm. Plainly the trypsin lowered the amount of coagulable protein by 3.4 mgm. A correction was not made, because 2 c. c. of the digestion mixture contained but 8 mgm. of trypsin, and the correction probably varied at different stages of digestion. This effect is regarded as the probable explanation of the apparent digestion in mixtures C, experiments 20 to 22.

To calculate the amount of coagulable protein digested past the coagulable stage, in experiment 22 for example, from the analytic data

in Table III, 1 c. c. of serum 268 in mixture A contained 97.5 mgm. of coagulable protein four days after the mixture had been prepared. In mixture D digestion was going on, and 1 c. c. of serum in this mixture contained but 4.7 mgm. of coagulable protein. The other 92.8 mgm., 95 per cent of the total, had been transformed into proteoses and peptones which do not coagulate on heating. This figure, 95 per cent, together with others similarly calculated, is to be found in Table VII.

AMINO NITROGEN.—Although the determination of coagulable protein digested past the coagulable stage, as described, gives valuable information regarding the extent of digestion, further information was obtained by determining the amino nitrogen liberated at the same time. Van Slyke's method and apparatus were used as described in a previous publication by the senior writer (*3, p. 696*). For analytic purposes ammonia was removed in the following manner: At the close of the digestion period 10-c. c. portions of the various mixtures were transferred to porcelain crucibles, to each of which 0.5 c. c. of concentrated hydrochloric acid was added to destroy the trypsin at the desired time. These were allowed to stand for one hour and then made faintly but distinctly alkaline to litmus strips by the addition of about 0.3 gm. of sodium carbonate. The crucibles were then placed in a vacuum desiccator provided with a shallow dish containing some *N*/5 sulphuric acid. No attempt was made to estimate the ammonia. That the removal was complete was shown by numerous blanks with pure ammonium sulphate in this and in the following procedure. The next day the contents of the crucibles were used for the amino-nitrogen determinations; 5 or 10 c. c. of digestion mixture were used for one determination, according to the amount of amino nitrogen present. The results are given in Table III, last column, and have been corrected for amino nitrogen in the trypsin and pepsin. The amounts found in mixture A represent the preformed amino nitrogen present in the serum or antitoxin, and were subtracted from the amounts found in the other mixtures, when calculating the amount liberated by the digestion.

For total amino-nitrogen determination, 10 c. c. of serum or antitoxin were boiled with 10 c. c. of concentrated hydrochloric acid for 24 hours under a reflux condenser. The mixture was evaporated to dryness to expel hydrochloric acid, made alkaline with sodium-carbonate solution, keeping the total volume small, and set aside in an incubator room for 24 to 48 hours for the ammonia to escape. The mixtures usually evaporated almost to dryness. They were taken up with water and 0.5 c. c. concentrated hydrochloric acid, transferred to a 50-c. c. volumetric flask and diluted to the mark. For a single determination in the amino-nitrogen apparatus, 5 or 10 c. c. of the unfiltered solution were used, containing 1 or 2 c. c., respectively, of hydrolyzed serum or antitoxin.

Pepsin-acid mixtures were introduced directly into the Van Slyke apparatus without removal of ammonia, as the amounts were too small

to have an appreciable influence. The determinations of coagulable protein and amino nitrogen in trypsin mixtures were made within a day or two of one another, but in the peptic mixtures a longer interval was regarded as permissible on account of the much slower liberation of amino nitrogen. The results are tabulated below. The amounts of amino nitrogen per gram of coagulable protein are practically the same in tetanus serum, anthrax serum, and ordinary beef and veal muscle tissue.

Milligrams of amino nitrogen per gram of coagulable protein

Tetanus antitoxin, 420 F	117.4
Tetanus serum, 374	133.2
Tetanus serum, 268	120.8
Average of 3	123.8
Tetanus antitoxin X[a]	123.8
Anthrax serum, 48	110.3
Anthrax serum 48.3	120.2
Average of 5	120.4
Normal beef or veal muscle tissue (*Berg, 3, p. 680*). On the basis of 16 per cent of nitrogen in protein	116.8
Above tissue freed from extractives	120.0

Banzhaf, Sugiura, and Falk (*1*) made analyses and determined the nitrogen distribution of a number of antiserums, but found no marked differences in the compositions of the proteins in normal serum, tetanus, and diphtheria globulins, etc. The above figures are in accord with the findings of these investigators. All indicate that the normal serum proteins, as they pass over into immune serum proteins, do not undergo chemical changes that are detectable by present methods. The same is probably true of tissue proteins.

To calculate the percentage of the total amino nitrogen liberated by digestion, in experiment 22, for example, from the analytic data in Table III, 1 c. c. of serum 268 in mixture D contained 2.73 mgm. of amino nitrogen after 5 days' digestion; at the same time a similar quantity of serum in mixture A, in which no digestion was taking place, contained 0.56 mgm. Therefore, in mixture D, 2.73–0.56 = 2.17 mgm. of amino nitrogen were liberated by the digestion for each cubic centimeter of serum. If all of the amino nitrogen were thus liberated, 11.78 mgm. would be present; hence, 2.17/11.78 = 18 per cent of the total was liberated by digestion. This figure, 18 per cent, together with other figures similarly calculated, is to be found in Table VII.

INOCULATION TESTS

The amounts of antitoxin remaining in the various mixtures were determined, after suitable digestion periods, according to the method described by Rosenau and Anderson (*13*).

[a] For antitoxin X, a lack of material for analysis necessitated the assumption of the above average.

AN EXAMPLE OF A TEST.—Experiment 5. On May 4, 1917, mixtures containing tetanus serum 374 were prepared as detailed in Table III. The same mixtures were used in experiments 4, 5, and 5.1. Chemical data were obtained 6 and 17 days after digestion began. On the day of the inoculation, 18 days after digestion began, a 0.5-c. c. portion of each mixture was diluted to 20 c. c. with salt solution, resulting in five dilute antitoxin solutions, all of which contained 1 unit per cubic centimeter if none of the antitoxin had been destroyed. Of course, no antitetanus units were present at any time in mixture E. Measured quantities of these diluted mixtures were then mixed with salt solution and tetanus-toxin solution as detailed in Table IV, which is a typical one. As three guinea pigs were used on each dose, 3 or 4 doses were prepared; later but 2 guinea pigs on each dose were used, beginning with experiment 6.

TABLE IV.—*Composition of the doses injected (experiment 5)*

[0.50 c. c. of digestion mixture was diluted to 20.0 c. c. with physiological salt solution; 1 c. c. of diluted mixture contained 1 unit of antitoxin if none had been destroyed]

Mixture.	Diluted mixture.	Salt solution.	Toxin solution.	Injected into 1 guinea pig.	Dose originally contained.
	C. c.	*C. c.*	*C. c.*	*C. c.*	*Unit.*
Mixture A	0.07	0.93	1.00	2.00	0.07
	.10	.90	1.00	2.00	.10
	.20	.80	1.00	2.00	.20
Mixtures B, C, D	.10	.90	1.00	2.00	.10
	.20	.80	1.00	2.00	.20
	.40	.60	1.00	2.00	.40
	.80	.20	1.00	2.00	.80
Mixture E	.80	.20	[a] 1.00	2.00	.00

[a] In all experiments after this, but 0.02 c. c. of toxin solution was mixed in the dose of mixture E for 1 guinea pig.

It was possible that the trypsin and sodium carbonate or pepsin and hydrochloric acid at their final dilutions might destroy 50 or 60 MLD (minimal lethal dose) out of the 100 in the test dose, and still the mixture E guinea pigs would die within the standard time of 96 hours. But by giving this group of pigs but 2 MLD instead of 100, any destructive action on the toxin would have manifested itself. Out of 12 such guinea pigs in 6 experiments, 9 died within 96 hours, 1 within 120 hours, 1 within 7 days, and 1 lived, indicating that there was no significant destruction of toxin.

Two samples of tetanus toxin, labeled "F1" and "F4," were obtained from the Hygienic Laboratory of the United States Public Health Service. The test dose (100 MLD) was the same for both, 0.0007 gm. The toxins were kept in a Hempel sulphuric-acid desiccator evacuated to 3 mm. of mercury, in a refrigerator room at 1° C, in the dark.

The toxin and antitoxin were not mixed in separate syringes as practiced by Rosenau and Anderson; instead they were mixed in weighing

bottles (clean and sterile) having a capacity of about 50 c. c., height 50 mm., diameter 40 mm. These serve as excellent containers. Thus when two guinea pigs were to be injected with similar doses, three doses were prepared by mixing 3 c. c. of toxin solution and 3 c. c. of diluted antitoxin mixture. After standing for one hour in diffuse sunlight at room temperature the doses were injected, 2 c. c. into one guinea pig.

In the following tables (V–VI) the results of two typical inoculation tests are detailed. Although the tests closed at the expiration of 96 hours after inoculation, the guinea pigs were always kept under observation for an additional 96 hours. The time of death is recorded in the tables; when no such time is recorded, it means that the animals receiving that particular dose were alive seven or eight days after inoculation. Guinea pigs that died during the night, between 11 p. m. and 8 a. m., were recorded as having died at 8 a. m. Otherwise, dead guinea pigs seldom remained in their cages for more than one hour.

TABLE V.—*Destruction of antitoxin by trypsin and sodium carbonate (experiment 5)*

[Tetanus serum 374. Toxin F4. Digestion period, 18 days on day of injection. Total of 48 guinea pigs, in 16 groups of 3 on each dose]

Mixture A (serum only).		Mixture B (sodium carbonate).		Mixture C (trypsin).		Mixture D (sodium carbonate and trypsin).		Mixture E (sodium carbonate and trypsin).	
Unit.	Died.	Unit.	Died.	Unit.	Died.	Unit.	Died.	Unit.	Died.
	Hours.		*Hours.*		*Hours.*		*Hours.*		*Hours.*
0.07	114		25		31		31		20
	138	0.10	31	0.10	42	0.10	42	0.00	25
	192		42		42		42		31
			31		66		43		
.10	.....	.20	42	.20	66	.20	55		
			42		66		55		
			55				114		
.20	.....	.40	66	.40	.....	.40	138		
			66				144		
		.80	.....	.80	.....	.80	.....		

ANTITOXIN DESTROYED (PER CENT)

0	91	82	[a] 82	.....

COAGULABLE PROTEIN DIGESTED PAST COAGULABLE STAGE (PER CENT)

0	0	80	78	.....

AMINO NITROGEN LIBERATED BY DIGESTION (PER CENT)

0	0	57	35	.....

[a] For the method of calculating these percentages see p. 481, 483, 487.

TABLE VI.—*Destruction of antitoxin by pepsin and hydrochloric acid (experiment 21)*

[Tetanus antitoxin 420 F. Toxin F1. Digestion period, 4 days on the day of injection. Total of 36 guinea pigs in 18 groups of 2 on each dose]

Mixture A (antitoxin only).		Mixture B (hydrochloric acid).		Mixture C (pepsin).		Mixture D (pepsin and hydrochloric acid).		Mixture E (pepsin and hydrochloric acid).	
Unit.	Died.	Unit.	Died.	Unit.	Died.	Unit.	Died.	Unit.	Died.
	Hours.		*Hours.*		*Hours.*		*Hours.*		*Hours.*
0.07	46	0.10	30	0.07	49	0.10	30	0.00	66
	66		42		66		30		90
.10	114	.20	30	.10	94	.20	30	[b]	.....
	114		30		114		30		
		.30	30	.15	.....	.30	30		
			30				30		
		.40	30			.40	30		
			30				30		
		.60	30			.60	30		
			30				30		
		.80	30			.80	30		
			30				30		

ANTITOXIN DESTROYED (PER CENT)

0	Over 87.	0	Over 87	.....

COAGULABLE PROTEIN DIGESTED PAST COAGULABLE STAGE (PER CENT)

0	8	[a] 10	95	.....

AMINO NITROGEN LIBERATED BY DIGESTION (PER CENT)

0	0	0	17	.....

[a] See p. 481. [b] Only 2 MLD in 1 dose of E.

INTERPRETATION OF ANALYTIC DATA AND INOCULATION TESTS

The term "unit" or "immunity unit" is used in this work in the sense defined by Rosenau and Anderson (*13, p. 6*) as follows:

> The immunity unit for measuring the strength of tetanus antitoxin shall be ten times the least quantity of antitetanic serum necessary to save the life of a 350-gram guinea pig for ninety-six hours against the official test dose of a standard toxin furnished by the Hygienic Laboratory of the Public-Health and Marine-Hospital Service.

At the time this definition was made (1907) the toxin was the standard and the antitoxin a derived or secondary standard. According to this definition, it was immaterial whether the guinea pig died or lived after the 96 hours. At the time of this writing (1917) the Hygienic

Laboratory had slightly changed the definition, as follows (from a typewritten statement):

> The test dose of the tetanus toxin F is 0.0007 gram. This should kill a 350-gram guinea pig in about 96 hours, when mixed with one-tenth unit of antitoxin. The test dose contains approximately 100 minimal lethal doses.

According to this definition, the antitoxin is now the standard from which the toxin is derived, and it is not immaterial whether the guinea pig is alive or dead after 96 hours—the 0.1 unit as later defined specifies that the guinea pig shall not live more than 96 hours if the dose contain but 0.1 unit. Either definition used consistently would undoubtedly lead to the same conclusions in this work, since these are based upon comparisons made under similar conditions rather than upon absolute values. The older definition was used, as it seemed to be more convenient. All doses of antitoxin which protected a guinea pig for 96 hours were regarded as containing at least 0.1 unit. There was generally little need for fine distinctions, as few of the guinea pigs died just before the 96-hour limit. With few exceptions these guinea pigs were regarded as having received less than 0.1 antitoxic unit, according to the older definition. In the following statement the number of guinea pigs that died before the time indicated is shown. All of them had received the test dose of toxin plus varying amounts of antitoxin. Twelve guinea pigs that received only 2 MLD are not included.

Time after inoculation:	Number of guinea pigs dead.
20 to 24 hours	24
25 to 48 hours	131
49 to 72 hours	36
	[a] 191
73 to 96 hours	19
97 to 120 hours	17
121 to 144 hours	9
145 to 168 hours	3
169 to 192 hours	1
Total	240

CALCULATION OF THE PERCENTAGE OF THE TOTAL ANTITOXIN DESTROYED.—It will be noticed in Table V that the three guinea pigs (and three others) that received a quantity of serum 374 containing 0.07 unit were protected for more than 96 hours against the test dose of toxin. Consequently the quantity of serum calculated to contain 0.07 unit, on the basis of the manufacturer's statement that the serum contained 202 units per cubic centimeter (see Table II) actually contained at least 0.1 unit, the manufacturer apparently having liberally understated the

[a] Dead in 72 hours.

potency, partly to allow for deterioration with time and partly in the desire to furnish promptly the product when requested. In a similar way, antitoxin X and serum 268 were found to have potencies about 50 per cent higher than that stated. The other preparation used, antitoxin 420F, stated to contain 300 units per cubic centimeter, protected guinea pigs for 96 hours in 0.1 unit doses, but not in 0.07 unit doses (see Table VI). This preparation had received a final and correct standardization before shipment. Therefore all the doses in Table V should be multiplied by 10/7. In mixture B the only guinea pigs that lived more than 96 hours were those that had received a quantity of serum calculated to contain 0.80 unit if none of the potency had been destroyed. The results with mixture A show that this dose contained originally, at least, 0.80 times 10/7, or 1.14 units. At the time of injection this dose (B) contained at least 0.1 unit, because the guinea pigs were protected for over 96 hours. Hence, at most, the destruction was $\frac{1.14-0.1}{1.14}$, or 91 per cent. The guinea pigs that received the next lower dose—that is, 0.40 times 10/7, or 0.57 units—died in less than 96 hours; therefore this dose contained less than 0.1 unit, or, over 0.47 out of 0.57 unit was destroyed (82 per cent). Consequently in this experiment over 82 but less than 91 per cent of the antitoxin in mixture B was destroyed. The higher figure was used throughout.

In Table VI the results with mixture A show that the doses actually contained the number of units stated in the table. In mixture D, for example, the 0.8-unit dose contained less than 0.1 unit, or the destruction was more than $\frac{0.8-0.1}{0.8}$, or 87 per cent. It is believed that the figures for percentage of antitoxin destroyed may be incorrect by a few points, but this is not of importance.

COMPARISON OF ANTITOXIN DESTROYED WITH PROTEIN DIGESTED.—Twelve inoculation tests were made, but all are not recorded here in detail, because Tables V and VI are thoroughly typical of the others. A discussion of the results of experiments 5 and 21 will apply to the others, because of their general concordance. With the exception of experiments 1 and 2, which were preliminary, the remaining 10 experiments, in which 360 guinea pigs were used, are summarized in Table VII.

TABLE VII.—*Comparison of antitoxin destroyed with protein digested*

EXPERIMENTS 3–6.1 : TRYPTIC DIGESTION

Experiment No.	Antitoxin No.	Toxin No.	Digestion period on day of inoculation.	Mixture.	Antitoxin destroyed.	Coagulable protein digested past coagulable stage.	Amino nitrogen liberated by digestion.
			Days.		*Per cent.*	*Per cent.*	*Per cent.*
3	420 F.	F_4	13	[a] A	0	0	0
				B	87	4	.....
				C	75	57	40
				D	87	65	37
3.1	420 F.	F_4	119	A	0	.....	.....
				B	over 95	.....	.....
				C	75–87	.....	.....
				D	over 95	.....	.....
4	Serum 374.	F_4	8	A	0	0	0
				B	65	0	0
				C	65	56	53
				D	65	58	31
5	Serum 374.	F_4	18	A	0	0	0
				B	91	0	0
				C	82	80	57
				D	82	78	35
5.1	Serum 374.	F_4	76	A	0	.....	.....
				B	96	.....	.....
				C	82–91	.....	.....
				D	82–91	.....	.....
6	Antitoxin X.	F_1	9	A	0	0	0
				B	82	77	[b] 71
				C	0	0	0
				D	82	77	70
6.1	Antitoxin X.	F_4	45	A	0	.....	.....
				B	82–93	.....	.....
				C	0	.....	.....
				D	82–93	.....	.....

EXPERIMENTS 20–22 : PEPTIC DIGESTION

20	420F.	F_4	11	[c] A	0	0	.....
				B	over 87	0	.....
				C	0	[d] 9	.....
				D	over 87	96	.....
21	420F.	F_1	4	A	0	0	0
				B	over 87	8	0
				C	0	[d] 10	0
				D	over 87	95	17
22	Serum 268.	F_1	3	A	0	0	0
				B	over 96	2	4
				C	0	6	5
				D	over 96	95	18

[a] In these mixtures A contained only antitoxin; in addition to this the others contained: B, sodium carbonate; C, trypsin; D, sodium carbonate and trypsin.
[b] See page 481.
[c] In these mixtures A contained only antitoxin; in addition to this the others contained: B, hydrochloric acid; C, pepsin; D, pepsin and hydrochloric acid.
[d] See page 481.

OBSERVATIONS ON RESULTS OF EXPERIMENTS 5 AND 21

The following observations on the results of experiment 5 (Table V) are true of the other experiments in which trypsin was used.

MIXTURE A.—This mixture contained only the serum and was always used as the standard with which the other mixtures were compared. Such a mixture undergoes very little change on standing and may be regarded as having a constant chemical composition and potency for periods such as those indicated in Table VII. All the mixtures were prepared under conditions assuring sterility. Bacterial action in the mixtures was prevented by chloroform. The results of mixture A served to standardize the technic of the experiments. For example, if the toxin used had undergone a loss in toxicity, it would have been apparent from results with this mixture. One test was made with a standard tetanus antitoxin, T17, furnished by the Hygienic Laboratory. Twelve guinea pigs were used, two on each dose, each of which received 0.1 unit of the standard antitoxin plus the following doses of toxin: Toxin F4; 0.00065, 0.0007, and 0.00075 gm. Toxin F1: 0.00065, 0.0007, and 0.00075 gm. The results indicated that at the time of this test (experiment 4, May 18, 1917), the standard antitoxin and toxin were of standard strength, and the experimental technic used was correct.

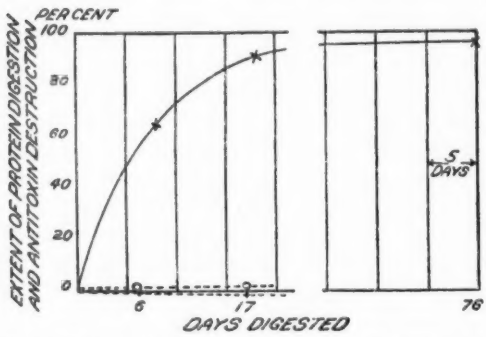

FIG. 1.—The destruction of tetanus antitoxin (—— × —) in 0.5 per cent sodium-carbonate solution without any change in total coagulable protein (— —○— —) or amino nitrogen (— +——). Mixture B, experiments 4–5.1.

MIXTURE B.—This contained serum in 0.5 per cent sodium-carbonate solution. (The mixture B for experiment 6–6.1 contained antitoxin X in a final concentration of *N/215* sodium hydroxid instead of the carbonate. The two solutions are equally alkaline.) In this mixture the antitoxin was slowly but completely destroyed by the sodium carbonate (or hydroxid). After 18 days' contact between the antitoxin and alkali (experiment 5) 91 per cent of the antitoxic units had disappeared; but during this time the data for total coagulable protein and amino nitrogen indicate that the protein contents of this mixture underwent no change. It would appear that in this way the antitoxin may be separated from the associated protein, but during the separation the antitoxic units are destroyed, leaving the proteins intact. This effect was not recorded by

any of the previous investigators mentioned on page 473; it probably was not looked for.

The figures in Table II for mixture B, experiment 5, are shown graphically in figure 1. From such data, if none other were available, the conclusion would be justified that the antibodies were separate from the proteins, so far as one may be almost totally destroyed without any detectable difference in the other.

MIXTURE C.—This mixture contained serum and trypsin. The original object was to make certain that the trypsin was immunologically neutral under the experimental conditions. The mixture was faintly amphoteric to litmus-paper strips. In the experiment 3 mixture it was faintly acid. The digestion in mixture C was as rapid as in mixture D, which contained 0.5 per cent of sodium carbonate and trypsin. In 18 days, in mixture C, 80 per cent of the coagulable protein was digested past the coagulable stage and 57 per cent of the total amino nitrogen was liberated. During this same time, 82 per cent of the antitoxin units were destroyed. The experimental data are graphically represented in figure 2. Because the rate of protein splitting is practically the same as the rate of antitoxin destruction in such a mixture, the supposition that the antitoxin and the protein are identical substances would be a reasonable one were it not for the fact that the data with mixture B show that the antitoxin may be destroyed without protein splitting.

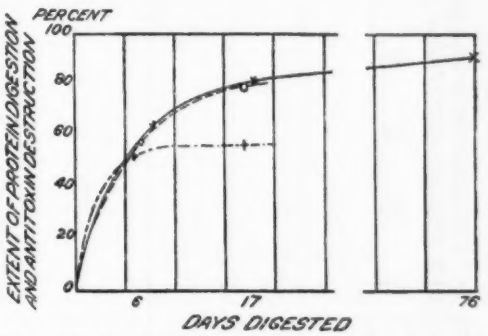

FIG. 2.—The destruction of tetanus antitoxin (——×——) by trypsin in solution amphoteric to litmus strips; the digestion of coagulable protein past the coagulable stage (— —○— —); the liberation of free amino nitrogen (—— ——+—— ——). Mixture C, experiments 4–5.1.

MIXTURE D.—The results were practically the same as with mixture C.

MIXTURE E.—This contained trypsin and sodium carbonate in the same quantities as mixture D, but no tetanus serum. Instead anthrax serum was used; in other E mixtures normal horse serum was used. This mixture obviously contained no tetanus antitoxic units, and was used for the purpose of ascertaining with certainty that the tetanus toxin was not destroyed by the largest amounts of trypsin and sodium carbonate in doses of other mixtures. (See page 484.) The highest final concentration of sodium carbonate in the toxin-antitoxin mixture injected was 0.008 per cent.

The following observations on the results of experiment 21 (Table VI) are true of the other experiments in which pepsin was used.

MIXTURE A.—This contained only antitoxin 420 F and was used as a standard with which the other mixtures were compared.

MIXTURE B.—This contained the antitoxin in 0.2 per cent hydrochloric-acid solution. From the data in Tables III, VI, and VII it is apparent that the acid destroyed all or very nearly all of the antitoxin in four days, during which time the proteins underwent practically no change. There was a slightly smaller amount of coagulable protein in mixture B than in mixture A, but in the other two B mixtures (experiments 20, 22) the differences were insignificant. The figures for amino nitrogen likewise indicate that while over 87 per cent of the antitoxin was destroyed, the changes in the proteins, as measured chemically, were so slight as to justify the conclusion that under these particular experimental conditions the destruction of the antitoxin involves no protein breakdown. The destruction of over 96 per cent of tetanus antitoxin in mixture B, experiment 22, without any significant change in the amounts of total coagulable protein or amino nitrogen are graphically represented in figure 3. This effect was not recorded by any of the previous investigators mentioned on page 473; it probably was not looked for. This affords a second method of separating tetanus antitoxin from associated protein; but the antitoxin is destroyed, leaving the antitoxin-free proteins intact.

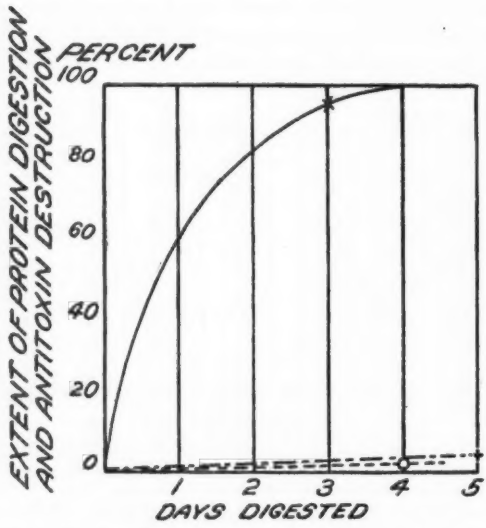

FIG. 3.—The destruction of tetanus antitoxin (—×—) by 0.2 per cent hydrochloric acid without any significant change in the amounts of total coagulable protein (— —○— —) or free amino nitrogen (— —+— —). Mixture B, experiment 22.

MIXTURE C.—There were no changes in this mixture containing antitoxin and pepsin—that is, there was neither antitoxin destruction nor protein digestion. The lower figures for total coagulable protein, as compared with mixture A, do not indicate a slight proteolysis, but rather a difficulty of obtaining a complete precipitation of the protein in the

presence of the proteoses, peptones, etc., which make up the bulk of the trypsin and pepsin preparations used, as explained on page 481.

MIXTURE D.—The destruction of antitoxin by pepsin-hydrochloric acid in this mixture was obviously due to the hydrochloric acid, as the results for mixture B indicate. Furthermore, these indicate that the proteolysis in mixture D can not with certainty be regarded as the immediate cause of the antitoxin destruction.

Figure 4 shows the simultaneous antitoxin destruction and protein splitting in mixture D, experiment 22.

Carrière's (*4*) finding that pepsin-hydrochloric acid does not affect tetanus antitoxin is probably incorrect.

MIXTURE E.—This showed that the pepsin-hydrochloric acid did not destroy appreciable amounts of the tetanus toxin. The highest final concentration of hydrochloric acid in the toxin-antitoxin mixture injected was 0.005 per cent in experiment 22. In experiments 20 and 21 it was 0.0027 per cent. In these three experiments the dose of mixture E injected into one guinea pig contained but 2 ML.D. The deaths of the guinea pigs resulted in the following number of hours after the subcutaneous injection of the mixture: 66, 66, 66, 72, 74, 90.

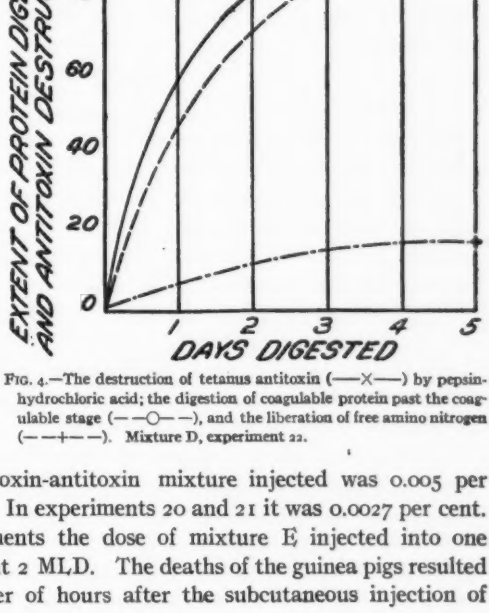

FIG. 4.—The destruction of tetanus antitoxin (—×—) by pepsin-hydrochloric acid; the digestion of coagulable protein past the coagulable stage (— —○— —), and the liberation of free amino nitrogen (— —+— —). Mixture D, experiment 22.

DESTRUCTION OF ANTHRAX IMMUNE BODIES BY PROTEOLYTIC ENZYMS

Before the writers began the work on tetanus serum, they were engaged in a similar study of anthrax serum. Inoculation experiments were made for the purpose of ascertaining whether the immune bodies in anthrax serum would be destroyed when the serum proteins were digested by pepsin-hydrochloric acid or trypsin-sodium-carbonate. The general method of the inoculation of the guinea pigs, the standardization of the virus, etc. have been described in a previous publication (*6, p. 41*). Chemical analyses were made, similar to those mentioned in the foregoing pages, for the purpose of measuring the extent of pro-

tein splitting and then comparing it with the loss in potency of the serum. The results obtained after 11 inoculation experiments, in which 371 guinea pigs were used, were inconclusive. The digested anthrax serum apparently immunized guinea pigs against anthrax virus as often as it did not. Repeated attempts to obtain definite results were unsuccessful. The main reason for this probably lies in the fact that there is no known quantitative relation between anthrax serum and virus such as there is between tetanus and diphtheria antitoxin and toxin. Unlike the unorganized tetanus and diphtheria toxins, which can not grow or multiply when injected into a test animal, a most carefully standardized dose of anthrax virus, containing a definite number of organisms, can grow to an extent which varies with the resistance of the animal.

SUMMARY

(1) Tetanus antitoxin in 0.5 per cent sodium-carbonate solution was slowly and completely destroyed. At the same time no significant chemical changes in the proteins were detected.

(2) In solutions amphoteric or faintly acid to litmus-paper strips, trypsin destroys the antitoxin, and at the same time the associated proteins are digested. The rates of antitoxin destruction and protein splitting were substantially the same.

(3) The results were the same with solutions containing trypsin and 0.5 per cent sodium carbonate.

(4) Tetanus antitoxin in 0.2 per cent hydrochloric acid was completely destroyed in three or more days. During this time no significant chemical changes in the proteins were detected.

(5) In neutral solutions pepsin did not affect the antitoxin.

(6) In pepsin-hydrochloric acid, proteolysis and antitoxin destruction proceed simultaneously.

(7) These results tend to indicate that tetanus antitoxin is a substance of nonprotein nature. But the stability of the antitoxin is so dependent upon that of the protein to which it is attached, that whenever the protein molecule is split, the antitoxin splits with it.

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RELATION OF THE DENSITY OF CELL SAP TO WINTER HARDINESS IN SMALL GRAINS[1]

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INTRODUCTION

Theoretical considerations suggest a close relation between the density of the cell sap and the ability of plants to survive low temperatures. If death from cold be due to the formation of ice in the plant tissue, to physiological drouth, to the precipitation of the proteids of the protoplasm, or the dessiccation of the protoplasm, then an increase in the electrolytic contents of the sap would increase the hardiness either by lowering the freezing point of the sap or by reducing transpiration.

These considerations led to a study of the sap density of various small grains as one phase of a series of investigations conducted to determine the causes of winter-killing. The work was done during the fall and winter seasons of 1915–16 and 1916–17. Practically all of the actual determinations were made by the junior writer.

EXPERIMENTAL METHODS

The experiments reported in this paper were confined to the winter cereals, rye, wheat, emmer, barley, and oats, which, as shown by general field experience, decrease in comparative winter hardiness in the order named.

Much time was consumed in working out a suitable technic, especially for the extraction of the cell sap. Maceration of the tissue is generally unsatisfactory without a means for centrifuging the sap. As pointed out by Dixon and Atkins,[2] the freezing point of sap extracted without previous treatment of the tissue is likely to be too low. Liquid air was not available for rendering the plasma membrane permeable, as suggested by Dixon and Atkins; hence, we resorted to chemical reagents. For this purpose toluene and chloroform vapor were employed.

This method is not entirely satisfactory because of the slight solubility of these reagents and the opportunity for changes in the cell sap during the rather long period of treatment that is necessary. However, it is believed the methods finally worked out circumvent these objections in a large degree, and that the experimental errors involved are too small to affect the conclusions reached in this investigation.

All determinations were from the leaves of plants sowed the last week in September or the first week in October. The samples were gathered in duplicate, placed immediately in air-tight cans, and taken to the laboratory. All lots for any one experiment were gathered at the

[1] Contribution 15 from the Department of Agronomy, Kansas Agricultural Experiment Station.

[2] DIXON, H. H., and ATKINS, W. R. G. OSMOTIC PRESSURES IN PLANTS. I. METHODS OF EXTRACTING SAP FROM PLANT ORGANS. *In* Notes Bot. School, Trinity Coll., Dublin, v. 2, no. 4, p. 154–176. 1913.

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same time of day and with the same conditions, as far as possible. In all cases, except when otherwise noted, the leaves were placed on ice or in a refrigerator at a temperature of 4° to 5° C. The extracted sap was kept on ice in tightly stoppered bottles, or in a refrigerator until a determination of the freezing point was made.

The freezing point was determined with a Beckman thermometer and the usual freezing-point apparatus, except that a tube somewhat smaller than usual was employed so that only a small quantity of sap (5–6 c. c.) was necessary for a determination.

The sap was extracted by pressure applied to the leaves which were first macerated, or treated with chloroform or toluene, as indicated.

EFFECT OF TIME OF TREATMENT ON DENSITY OF EXTRACTED SAP

In practically all cases in which different varieties or kinds of grain were compared, determinations were made in duplicate, and in nearly all cases considerable variation between duplicate samples was found. At first this was thought to be due to imperfect extraction of the sap. Since the sap first expressed has a lower density than that extracted later, as shown by Dixon and Atkins and confirmed by us, imperfect extraction might have considerable effect on the density. To avoid this error, experiments were conducted in which the leaves were treated for various periods with chloroform or toluene. Wheat (*Triticum* spp.) leaves were used in the experiments with chloroform, and wheat and barley (*Hordeum* spp.) leaves in those with toluene. In part of the experiments with chloroform duplicate samples were kept during treatment at both room temperature and in an ice box at a temperature of 4° to 5° C. The results are shown in Tables I and II.

TABLE I.—*Effect on sap density of treating wheat with chloroform for different periods and at different temperatures*

Period of exposure.	Freezing point of sap after treatment (°C.).				
	Room temperature.	Room temperature.	Ice box.	Room temperature.	Ice box.
Hours.					
0.25	−0.638	.....	.....	.....	.....
.5	−1.205	.....	.....	.....	.....
1.0	−1.325	−1.325	−0.913	.....	.....
1.5	−1.510	.....	.....	.....	.....
2.5	.....	−1.525	−1.400	.....	.....
4.0	.....	−1.600	−1.568	.....	−1.520
5.5	.....	−1.675	−1.545	.....	−1.770
15.5	.....	.....	.....	−1.89	−1.510
21.5	.....	.....	.....	.....	−2.082
23.0	.....	.....	.....	−2.263	−2.170
39.5	.....	.....	.....	−2.040	−1.475
47.0	.....	.....	.....	−1.895	−1.320
63.5	.....	.....	.....	−1.575	−1.940
71.0	.....	.....	.....	−1.396	−2.030
87.5	.....	.....	.....	−1.340	−2.090
95.0	.....	.....	.....	−1.725	−2.035

TABLE II.—*The effect on sap density of treating leaves of barley and wheat with toluene for different periods*

Exposure to toluene.	Freezing point of sap after treatment (°C.).					
	Barley.			Wheat.		
	Sample 1.	Sample 2.	Average.	Sample 1.	Sample 2.	Average.
Hours.						
14–15	−1.295	−0.975	−1.135	−1.230	−1.040	−1.140
22.5–23	−1.515	−1.565	−1.540	−1.575	−1.670	−1.623
37.5–38	−1.310	−1.355	−1.333	−1.335	−1.275	−1.305
46–46.5	[a]−1.725	[a]−1.710	−1.718	−1.320	−1.395	−1.385
61.5	−1.350	−1.315	−1.333	.....	.....	.....

[a] The freezing point of these samples was not determined until several hours after extraction. Molds had then appeared on the sap, and this is perhaps responsible for the large depression.

The results show a gradual increase in the depression of the freezing point with exposure to chloroform and toluene up to about 23 hours.

This would indicate that the plasma membrane had been rendered thoroughly permeable at that time. There appeared to be no marked difference whether the leaves were kept on ice or at room temperature. Since enzymic action would be least at the low temperature, it was thought best to keep the samples on ice during treatment, and this was done in most cases.

EFFECT OF CHLOROFORM AND TOLUENE ON THE DEPRESSION OF THE FREEZING POINT

Since chloroform and toluene are slightly soluble in water, it seemed desirable to determine their effect on the freezing point of the sap as a possible explanation for the variation in duplicate samples. Accordingly, different amounts were added to distilled water, and the freezing point of the solutions determined. The results are presented in Table III.

TABLE III.—*Effect of chloroform and toluene on the freezing point of distilled water*

Solution.	Freezing point (°C.).	Solution.	Freezing point (°C.).
Distilled water	0.000	Toluene, 1 per cent.	−0.020
Chloroform, 1 per cent.	− .080	Toluene, 5 per cent.	− .038
Chloroform, 5 per cent.	− .100	Toluene, 10 per cent.	− .035
Chloroform, 10 per cent.	− .130		

The solubility of chloroform and toluene appears to be too low to affect the freezing point enough to explain the discrepancies observed in duplicate samples. The slight error due to variation in the amount taken up by the sap could scarcely have been an important factor in the results reported later in this paper. This particularly would be true

of those treated with toluene, its solubility being less than that of chloroform.

RELATIVE FREEZING POINT OF THE SAP OF DIFFERENT KINDS AND VARIETIES OF GRAIN

In this study different kinds of cereals known to vary widely in their resistance to winter killing were selected. The first determination was made on November 27, 1915. The leaves were gathered between 8.15 and 9 a. m. The day was cool and cloudy, with an occasional light shower. The leaves were treated with chloroform for 30 minutes. Probably the depression is somewhat less than it would have been had they been treated for a longer period. The sap was placed on ice as soon as extracted, and left until the freezing point was determined. The results are given in Table IV. The varieties are given in this and the following tables in the order of their hardiness as shown by general field experience.

TABLE IV.—*Relative freezing point of the sap of winter cereals on November 27, 1915*

Kind of grain.	Variety.	Freezing point of sap after treatment (°C.).		Average.
		Sample 1.	Sample 2.	
Rye (*Secale cereale*)		−0.985	−1.095	−1.044
Wheat	Kharkof	−1.230	−1.230	−1.230
Do	Fultz	− .982	−1.170	−1.076
Emmer (*Triticum dicoccum*)	Black Winter	− .990	−1.035	−1.012
Barley	Tennessee Winter	−1.125	−1.110	−1.117
Oats (*Avena sativa*)	Culberson Winter	−1.220	−1.178	−1.199

The data can scarcely be said to show a close relation between the sap density and hardiness. Kharkof wheat, which is very hardy, shows the greatest depression of the freezing point, but the depression for Culberson oats, which is the least hardy of any of the winter cereals, is almost as great. The depression for rye, which will survive greater degrees of cold than any other cereal, is much less than for winter oats.

A second determination was made on December 17 and 18, 1915. The leaves were gathered at 1.30 p. m. on the 17th, placed in air-tight cans, and taken to the laboratory. They were then macerated in a mortar, and the sap was filtered and placed on ice until the next day, when the freezing point of each sample was determined. December 17 was clear and cold. The air temperature when the leaves were gathered was −16.9° C. (1.5° F.) and the soil temperature at a depth of 1 inch was −6.1° C. (21° F.). The leaves of many of the plants were badly wilted, indicating inability to secure enough water from the cold and frozen soil to supply that lost by transpiration. The results are presented in Table V.

TABLE V.—*Relative freezing point of the sap of winter cereals on December 17, 1915*

Kind of grain.	Variety.	Freezing point of sap after treatment (°C.).		
		Sample 1.	Sample 2.	Average.
Rye		−1.330	−1.020	−1.175
Wheat	Kharkof	− .840	−1.030	− .935
Do	Fultz	−1.595	−1.300	−1.442
Barley	Tennessee Winter	−1.330	−1.300	−1.320
Oats	Winter Turf	−1.220	−1.300	−1.260
Do	Culberson	−1.470	−1.420	−1.445

As before, there seems to be little relation between the freezing point of the sap and the known ability of the different cereals to survive severe winters. The greatest depression is recorded for Culberson oats and the least for Kharkof wheat.

This may be explained by assuming that the oats are less able to secure sufficient water to supply that lost by transpiration. Hence, the water content of the tissue would be low and the cyroscopic value of the extracted sap high.

A third determination was made on January 10, 1916. The leaves were gathered at 8.30 a. m. The day was cool and cloudy. The soil was wet, but not frozen. The plants appeared to be turgid. The leaves were treated for 22.5 hours with toluene on ice (temperature 3° C.), allowed to stand exposed to the air for ten minutes to permit the excess of toluene vapor to leave the tissue, and then expressed. The sap was kept on ice at a temperature of about 1.0° C. until January 11, when the freezing point was determined. The results are given in Table VI.

TABLE VI.—*Relative freezing point of the sap of winter cereals on January 10, 1916*

Kind of grain.	Variety.	Freezing point of sap after treatment (°C.).		
		Sample 1.	Sample 2.	Average.
Rye		−1.450	.....	−1.450
Wheat	Turkey	−1.360	−1.510	−1.435
Do	Fultz	−1.390	−1.440	−1.415
Barley	Tennessee Winter	− .970	−1.390	−1.180
Oats	Winter Turf	−1.385	−1.525	−1.455
Do	Culberson	−1.540	−1.380	−1.460

In this case also there appears to be little if any relation between the freezing point of the sap and winter hardiness. In fact, with the exception of the barley, the freezing point of all is about the same. There is only 0.01 degree difference in the freezing point of the sap of rye, which is the hardiest of the cereal grains, and of Culberson oats, which is the least hardy.

A test comparing rye, wheat, and barley was conducted on March 8. The leaves were collected at 4.30 p. m. They were treated with toluene for about 16 hours, and the sap was kept on ice until the freezing point was determined. The results are given in Table VII.

TABLE VII.—*Relative freezing point of the sap of winter cereals on March 8, 1916*

Kind of grain.	Variety.	Freezing point of sap after treatment (°C.).		
		Sample 1.	Sample 2.	Average.
Rye		−1.250	−1.615	−1.433
Wheat	Kharkof	−1.945	−2.015	−1.980
Barley	Tennessee Winter	−1.710	−1.765	−1.738

Here also no significant differences were observed. The barley showed a greater depression than the rye, although much less able to survive low temperatures.

A fifth test compared wheat, barley, and oats grown in the greenhouse. The leaves were from plants about 40 days old from planting. They were watered several times before gathering, in order to insure a normal condition with respect to turgidity. The leaves were treated with toluene on ice for 16.5 hours, and the extracted sap was placed on ice until the freezing point was determined. The results, which are presented in Table VIII, show no large differences and apparently no relation between depression of the freezing point and the ability of the different kinds of grain to survive low temperatures.

TABLE VIII.—*Relative freezing point of the sap of winter cereals grown in the greenhouse*

Kind of grain.	Variety.	Freezing point of sap after treatment (°C.).		
		Sample 1.	Sample 2.	Average.
Wheat	Kanred	−1.700	.....	−1.700
Barley	Tennessee Winter	−1.840	−1.780	−1.810
Oats	Culberson	−1.595	−1.755	−1.675

Several determinations were made directly without extracting the sap. This was done by wrapping the thermometer bulb with leaves, placing it in the air chamber, and inserting the whole in the freezing mixture of salt and ice, and proceeding in the usual way. This method appears to have been used by Müller-Thurgau and more recently by Bouyoucos and McCool.[1]

[1] BOUYOUCOS, G. J., and MCCOOL, M. M. DETERMINATION OF CELL SAP CONCENTRATION BY THE FREEZING POINT METHOD. *In* Jour. Amer.-Soc. Agron., v. 8, no. 1, p. 30. 1916.

Owing to the time required to make readings, only two or three varieties could be examined at one time. The data are presented in Table IX. Each recorded reading is the average of several from the same sample.

TABLE IX.—*Relative freezing point of the leaves of winter cereals*

Date of determination.	Freezing point (°C.).			
	Kharkof wheat.	Fultz wheat.	Rye.	Barley.
January 16, 1917	.....	.....	−3.56	−2.59
January 19, 1917	−3.47	.....	−3.58	.....
January 27, 1917	−2.17	−2.06	−2.10	.....

The depression of the freezing point determined in this way is somewhat greater than for the extracted sap, but the differences between kinds of grain are not consistent enough to certainly indicate a relation between the freezing point of the sap and ability to resist cold. In two tests the rye has a lower freezing point than either barley or wheat; but in a third test, on January 27, Kharkof winter wheat showed a greater depression than the rye.

An attempt was made to obtain a measure of the accuracy of this method by collecting four samples of winter wheat at the same time from the same plot, and treating them identically. The freezing point of each is shown in Table X.

TABLE X.—*Freezing point of duplicate samples of wheat determined by the direct method*

Sample No.	Freezing point (°C.).
1	−3.66
2	−3.85
3	−3.57
4	−3.53
Average	−3.65

It will be seen that the variations are of the same order as those observed in determinations of the extracted sap.

Table XI summarizes the data from all tests. Since the depression of the freezing points appears to depend so much on the balance the plants are able to maintain between absorption and transpiration, it would manifestly be unfair to include any tests but those in which the leaves were secured under like conditions. Hence, the average includes the first three tests only.

TABLE XI.—*Summary of the relative freezing points of the leaves and leaf sap of various winter cereals*

Date.	Freezing point (° C.)						
	Rye.	Kharkof wheat.	Fultz wheat.	Black Winter emmer.	Tennessee Winter barley.	Winter Turf oats.	Culberson Winter oats.
Nov. 27, 1915..	−1. 044	−1. 230	−1. 076	−1. 012	−1. 117	.....	−1. 199
Dec. 17, 1915..	−1. 175	− . 935	−1. 442	.....	−1. 320	−1. 260	−1. 445
Jan. 10, 1916...	−1. 450	−1. 435	−1. 415	.....	−1. 180	−1. 455	−1. 460
Mar. 8, 1916...	−1. 433	−1. 980	.....	.....	−1. 738	.....	.....
Jan. 16, 1917...	−3. 560	.....	.....	.....	−2. 590	.....	.....
Jan. 19, 1917...	−3. 580	−3. 470	.....	.....	.....	.....	.....
Jan. 27, 1917...	−2. 100	−2. 170	−2. 060	.....	.....	.....	.....
Average [a]..	−1. 223	−1. 200	−1. 311	.....	−1. 208	.....	−1. 334

[a] Average of determinations of November 27, December 17, and January 10, only.

The average for the three tests show practically the same depression of the freezing point for rye, Kharkof wheat, and Tennessee Winter barley, it being decidedly less for these grains than for Fultz Winter wheat and Culberson Winter oats. As mentioned in connection with the data for each test, the depression of the freezing point seems to depend more on the conditions of the leaves with respect to turgidity than on variety characteristics.

RELATION OF TURGIDITY TO DENSITY OF SAP AND RESISTANCE TO LOW TEMPERATURE

Because of the apparent relation between turgidity and sap density observed in these experiments, it seemed desirable to investigate this phase more thoroughly. Accordingly seedlings of hard wheat (Turkey), soft wheat (Fultz), and winter oats (Culberson), grown in the greenhouse were removed from the soil and one set was exposed to sunlight at room temperature from two to three hours and another set placed in the same location as the first but with the roots immersed in water. The freezing point of the leaves was then determined by the direct method. The results are shown in Table XII.

TABLE XII.—*Relation of turgor to the freezing point of leaves*

Variety.	Freezing point (°C.).					
	Turgid.			Wilted.		
	No. 1.	No. 2.	Average.	No. 1.	No. 2.	Average.
Hard wheat (Turkey)....	−1. 280	−1. 630	−1. 405	−1. 91	−1. 98	−1. 945
Soft wheat (Fultz).....	−1. 510	−1. 105	−1. 308	−1. 93	.....	−1. 930
Winter oats.....	−1. 180	−1. 280	−1. 230	−2. 34	−1. 98	−2. 160
Average.....	.....	.....	−1. 314	.....	.....	−2. 012

The results show beyond doubt the effect of turgidity on the freezing point of the extracted sap, the difference between wilted and turgid leaves being decidedly greater than was observed between different kinds of grain.

Further evidence is furnished by experiments conducted during the winter of 1915–16 in which wilted and turgid plants were exposed for different periods to freezing temperatures. Lots of 15 plants each were treated in four different ways before exposure to cold as follows: (1) Exposed to air for 30 minutes, (2) kept in air-tight cans for 30 minutes, (3) roots in water for 10 minutes, and (4) entire plant submerged in water for 10 minutes. All plants were then placed in a chamber surrounded with salt and ice in which the temperature was from $-2.0°$ to $-3.0°$ C. Portions of each lot were removed at intervals of 10, 20, and 30 minutes and placed with their roots in water to test their ability to recover. The results are shown in Table XIII.

TABLE XIII.—*Relation of turgidity to cold resistance*

Period of freezing.	Effect on plants.			
	Lot 1, exposed to air.	Lot 2, kept in air-tight cans.	Lot 3, roots in water.	Lot 4, plants submerged in water.
Minutes.				
10	Not injured	Not injured	Slightly injured	Slightly injured.
20	do	Slightly injured	Killed	Killed.
30	Slightly injured	Killed	do	Do.

In all cases the plants which were submerged in water or whose roots were in water were killed or injured decidedly more than those which were exposed to the air previous to freezing.

In another experiment conducted in a similar manner the plants were exposed to the air at greenhouse temperature for different periods of time and then exposed to a temperature of $-6.5°$ C. for about 10 minutes. One set was exposed to the air for 2.5 hours, another for 1.5 hours, another for 1 hour, and another for 30 minutes before freezing. Plants which had not been exposed to the air and others that had been exposed to the air for 2.5 hours but which had not been frozen were included as controls. All which had been wilted for 1.5 hours or longer survived with no apparent injury, while all turgid plants and those wilted for 1 hour or less were entirely killed. The appearance of typical plants from each lot after treatment is shown in Plate 53.

SUMMARY

To summarize briefly, there appears to be no relation between the cryoscopic value of the extracted sap of winter rye, wheat, emmer, barley, and oats grown in the field with normal conditions and their

ability to resist winterkilling. Turgidity of the tissue as influenced by physiological drouth appears to have more influence than the kind of grain on the concentration of the cell sap.

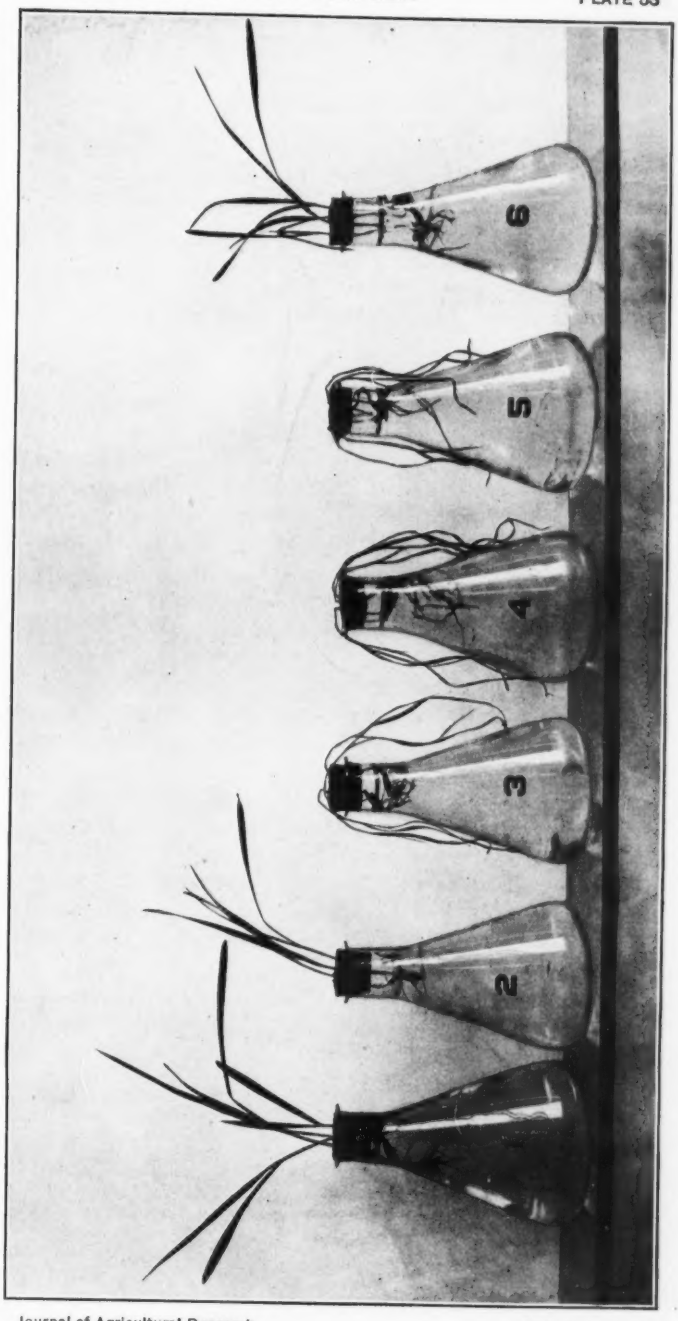
On the other hand, for tender plants of the same varieties grown in the greenhouse there appears to be a definite relation between the freezing point of the cell sap, the turgidity of the tissue, and resistance to low temperature. This relation for plants grown in the field perhaps also holds true in the fall and early winter and following periods of mild weather during the winter.

XUM

PLATE 53

Effect of wilting on ability of small grains to survive low temperatures:

Flask 1.—Exposed to air for 2.5 hours previous to freezing.
Flask 2.—Exposed to air for 1.5 hours previous to freezing.
Flask 3.—Exposed to air for 1 hour previous to freezing.
Flask 4.—Exposed to air for 0.5 hour previous to freezing.
Flask 5.—Not exposed to the air previous to freezing.
Flask 6.—Exposed to the air for 2.5 hours, but not frozen.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INFLUENCE OF TEMPERATURE AND PRECIPITATION ON THE BLACKLEG OF POTATO

By J. ROSENBAUM, *Assistant Pathologist, Cotton, Truck, and Forage Crop Disease Investigations, Bureau of Plant Industry, United States Department of Agriculture,* and G. B. RAMSEY, *Assistant Pathologist, Maine Agricultural Experiment Station*

OVERWINTERING IN THE SOIL

It is still a question in the minds of some pathologists whether the blackleg organism,[1] *Bacillus phytophthorus* appel, can remain in the soil over a winter and start the disease the following spring. In order to test this point, about a bushel of potato tubers (*Solanum tuberosum*), badly diseased with the blackleg, were collected and placed at the usual depth, about 2 or 3 inches apart, on October 11, 1915, in Aroostook County, Maine. In the spring of 1916, at the time when the general crop of potatoes was being planted, some Irish Cobbler and Spaulding Rose tubers were carefully selected, disinfected in the usual manner, and planted in the rows where the diseased potatoes had been planted the previous fall. When these were being planted a large number of overwintered tubers were found in the soil, either wholly or partially decayed. A careful watch was kept of the plants throughout the growing season. In neither the Irish Cobbler, an early-maturing variety, nor the Spaulding Rose, a late-maturing variety, was a single case of blackleg developed.

In the fall of 1916 a similar experiment was begun. The same soil was used, but instead of naturally infected tubers being used, approximately 3 pecks of Irish Cobbler potatoes were inoculated by means of a hypodermic syringe with a very virulent culture of the blackleg organism. The inoculated tubers were then placed in a covered container and allowed to remain there until each of them showed a marked rot at the point of inoculation. The tubers were then planted whole, about 1 foot apart in a trench 5 inches deep. On May 18,1917, this row was opened with a hand hoe, care being taken not to disturb the decayed tubers any more than necessary. Healthy Irish Cobbler potatoes, known to be free from blackleg, were cut in the usual way, and one seed piece was placed in the old seed bed with each of the decayed, overwintered tubers. A second row was made by the side of the one mentioned and planted with the same

[1] The blackleg organism has been generally referred to as *B. phytophthorus* Appel. According to the works of Morse (MORSE, W. J. STUDIES UPON THE BLACKLEG DISEASE OF THE POTATO, WITH SPECIAL REFERENCE TO THE RELATIONSHIP OF THE CAUSAL ORGANISMS. *In* Jour. Agr. Research, v. 8, no. 3, p. 79–126. 1917.) all cultures of blackleg isolated by him in Maine agree with *B. solanisaprus* Harrison, but for certain reason, he believes the name *B. atrosepticus* Van Hall should be adopted. According to the work of Smith (SMITH, Erwin F. BACILLUS PHYTOPHTHORUS APPEL. *In* Science, v. 31, no. 802, p. 748–749. 1910.), *B. phytophthorus* and *B. solanisaprus* are different.

kind of healthy seed to serve as a control. Each of these rows was 110 feet long. No blackleg showed in the plants during the growing season, and at digging time, September 10, the tubers were healthy and normal in every respect.

A similar experiment was carried out at Norfolk, Va.[1] Half a barrel of artificially infected tubers were thoroughly rotted by the blackleg organism before they were placed in the soil. There is no question that the bacteria were introduced into the soil in large numbers. The following spring when the potatoes were planted, some of the rotted tubers were still to be found in the soil. No blackleg appeared throughout the course of the experiment.

As supplementary tests to ascertain whether the organism still remained alive in the soil where the diseased tubers were planted, in each of the three experiments mentioned above isolations were made from the tubers themselves and in two of the experiments from the soil immediately surrounding the tubers. Many series of these isolations were made from various parts of the plots, and a great number of bacteria were obtained. All of these were tested for their ability to produce blackleg by innoculating healthy, growing plants and cut slices of healthy tubers. As a control for these inoculations, two different strains of authentic cultures of blackleg were used. The results of this work showed that the bacteria isolated from the soil were unable to produce the disease, while the two control cultures did produce the typical blackleg symptons.

In no case, therefore, was the blackleg organism found to live over a winter in the soil or in the tubers remaining in the soil. Morse [2] says:

> Observations in Maine indicate that under the climatic conditions which exist there infected seed potatoes are the sole source of infection and distribution and that the disease does not live over the winter in the soil.

In order to get an idea as to whether the results described above are normal, or whether the winter of 1916 was unusually severe, data in regard to the temperature, amount of snowfall, and total precipitation, were collected for three consecutive winters, as shown in Table I. Examination of this table shows that the winter of 1915–16 was not an unusual one. The average mean minimum and maximum during that season was slightly less than for the winter of 1914–15 but more than for that of 1913–14. The snowfall in inches in 1915–16 was considerably more than in 1914–15 and only slightly less than during 1913–14. This data would seem to indicate that the blackleg organism under the winter conditions that exist in northern Maine cannot survive in the soil or in the diseased tubers remaining in the soil.

[1] The work at Norfolk was done in cooperation with the Virginia Truck Experiment Station and was in charge of Mr. J. A. McClintock.

[2] Morse, W. J. Op. cit., p. 91.

TABLE I.—*Temperature and precipitation at Presque Isle, Me., 1913–1916*

Month.	1913–14				1914–15				1915–16			
	Temperature.		Precipitation.		Temperature.		Precipitation.		Temperature.		Precipitation.	
	Mean.				Mean.				Mean.			
	Minimum.	Maximum.	Snow.	Total.	Minimum.	Maximum.	Snow.	Total.	Minimum.	Maximum.	Snow.	Total.
	°F.	°F.	*Inches.*	*Inches.*	°F.	°F.	*Inches.*	*Inches.*	°F.	°F.	*Inches.*	*Inches.*
November	22	44	2.65	0.71	18.5	36.8	12	3.5	24.2	38.3	9	2.19
December	13	27	27	3.49	4.4	24.1	18	2.08	14.3	28.5	33.5	4.05
January	−9.4	17.1	29	2.95	2.0	23.5	17.5	2.75	−.67	21.5	21	2.2
February	−13.1	16.4	23	2.7	6.3	28.8	22	4.1	−1.1	20.6	18.5	2.35
March	16.5	34.7	15	2.5	13.8	33.2	13	1.4	4.4	28.7	11	1.1
April	19.4	44.3	25	4.0	29.3	49.7	Trace	3.4	29.1	53.4	17	1.75
Average	8.06	30.58	20.27	2.72	12.38	32.68	13.74	2.87	11.70	31.8	18.3	2.27

The experiment at Norfolk also seems to indicate that the organism does not overwinter in the soil, even under climatic conditions much milder than those found in Maine.

BLACKLEG AS FOUND IN NORTHERN MAINE IN 1914, 1915, AND 1916

During the summer of 1914, following a winter with an average mean minimum temperature of 8.06° F., an average mean maximum of 30.58°, and an average monthly precipitation of 2.72 inches, the blackleg was very prevalent in the potato fields under observation in Aroostook County. It was then attributed to a variety of conditions. However, no actual counts showing the percentage of the disease were made. The following year (1915) blackleg was likewise very prevalent in the same and other fields. This was also true in commercial fields where disinfection but not careful selection of the seed was practiced. The Irish Cobbler and Spaulding Rose were attacked as severely as any of the other varieties. For this reason the observations recorded here for 1915 and 1916 have been made on these two varieties. During these two summers a number of fields were inspected, and counts made as to the percentage of infected plants. As far as possible, counts were made on the same fields in 1915 and 1916. In all cases the seed used in 1916 was the progeny of the fields from which the counts were made in 1915. The results are presented in Table II. The significant thing about the results is the great difference in the percentage of blackleg found during 1915 and 1916. There is a striking uniformity in regard to this difference in six different fields from two varieties, an early and a late one. The counts were made approximately on the same date in both summers, and as far as possible, the same treatment and cultural conditions were employed both seasons. By examining the percentages of disease in the totals as given in Table II for the Spaulding Rose, it will be seen that in 1915 the percentage of blackleg is 8.47, 7.52, and

7.77, as compared to 0.84, 1.96, and 1.00 per cent in 1916 for the three fields, an average difference of 6.65 per cent. The data for the Irish Cobbler show 4.04, 1.57, and 5.79 per cent in 1915, as compared to 2.1, 1.57, and 1.17 per cent in 1916 for the three fields, an average difference of 2.19 per cent.

TABLE II.—*Prevalence of blackleg of Irish potatoes at Presque Isle, Me., during 1915 and 1916*

SPAULDING ROSE

Date of count.	Field No.	1915			1916		
		Number of healthy hills.	Number of diseased hills.	Percentage of disease.	Number of healthy hills.	Number of diseased hills.	Percentage of disease.
June 30	1	224	6	2.61	1,500	12	0.79
July 14	1	165	22	11.76	1,490	15	1.00
August 23	1	162	23	12.43	300	1	.33
Total		551	51	8.47	3,290	28	.84
June 30	2	200	9	4.31	312	12	3.70
July 14	2	196	18	8.41	290	3	1.02
August 23	2	256	26	9.22	400	5	1.23
Total		652	53	7.52	1,002	20	1.96
June 30	3	200	15	6.98	600	12	1.96
July 14	3	297	27	8.33	480	2	.41
August 23	3	310	26	7.74	300	0	0
Total		807	68	7.77	1,380	14	1.00

IRISH COBBLER

		1915			1916		
June 30	1	290	6	2.03	916	12	1.29
July 14	1	312	14	4.29	400	18	4.31
August 23	1	300	18	5.66	317	5	1.55
Total		902	38	4.04	1,633	35	2.10
June 30	2	400	2	.50	400	4	.99
July 14	2	360	7	1.91	388	12	3.00
August 23	2	366	9	2.40	400	3	.74
Total		1,126	18	1.57	1,188	19	1.57
June 30	3	200	5	2.44	200	10	4.76
July 14	3	200	22	9.91	517	2	.39
August 23	3	300	16	5.06	300	0	0
Total		700	43	5.79	1,017	12	1.17
Total for 3 fields		2,728	99	3.5	3,838	66	1.69

In general, the results presented in Table II for the years 1915 and 1916 are wholly comparable and give one an idea as to the difference in the relative prevalence of blackleg throughout Aroostook County dur-

ing the two seasons. Aside from the counts as given, careful watch was kept of a large number of fields throughout the county and in all cases there was a surprisingly small amount of blackleg in 1916 as compared to 1915. This was especially true during the month of August. This also explains why there is a greater difference shown in the Spaulding Rose than in the Irish Cobbler. The former, being a late variety, generally shows a greater number of plants diseased with the blackleg in August than the latter. In 1915 the Spaulding Rose showed a number of affected plants during August, but in 1916 few or none were seen during the same month. With the Irish Cobbler, on the other hand, blackleg makes its appearance in the latter part of June and early July and usually continues in evidence up into the first part of August. By this time potatoes begin to approach maturity, and very few additional plants show symptoms of the blackleg.

The writers wish to call attention to one exception found in 1916. On a one-six-acre plot of Irish Cobbler tubers used in connection with another experiment, a portion showed approximately 8 per cent of blackleg during August. This part of the plot was very low and wet, compared with the adjoining plots, and, as will be seen later, this explains the large percentage of diseased plants.

TEMPERATURE AND PRECIPITATION DURING 1914, 1915, AND 1916.

Morse[1] makes the following statement regarding the influence of weather conditions on blackleg:

> The progress of the disease is markedly influenced by weather conditions. . . . . More blackleg is observed in wet than in dry seasons.

and further—

> Soil conditions also are factors which influence outbreaks of blackleg. All other things being equal, the disease is more likely to occur in wet than in dry soil, and is more prevalent when the early part of the growing season is characterized by abundant rainfall.

The great difference in the severity of the disease found in 1914, 1915, and 1916 can be explained by differences in temperature and precipitation found during the three growing months, June, July, and August, during these three years, especially as it existed in August. In Table III are given the mean maximum and the mean minimum temperature, and the precipitation. Averaging the three months, it is seen that both the mean maximum and the mean miminum temperatures in 1916 were higher than in 1915 or 1914, while the precipitation for the same year was less than in the two preceding years. During the month of August, which showed the greatest differences in the percentages of the disease, the mean maximum in 1916 was 79° F., while in 1915 it was 72.7°, a difference of 6.3°; the mean minimum in 1916 was 64.8° as compared to 50.9° in 1915, a difference of 13.9°. The higher maximum as well as the

[1] Morse, W. J. op. cit., p. 84-85.

higher minimum in 1916 as compared with 1915 during the month of August makes the actual difference in temperature much greater than is indicated by the above figures.

TABLE III.—*Temperature and precipitation records at Presque Isle, Me.*

Month.	1914.			1915.			1916.		
	Mean temperature.		Precipitation.	Mean temperature.		Precipitation.	Mean temperature.		Precipitation.
	Maximum.	Minimum.		Maximum.	Minimum.		Maximum.	Minimum.	
	°F.	°F.	*Inches.*	°F.	°F.	*Inches.*	°F.	°F.	*Inches.*
June	69.1	42.9	4.8	73.7	47.6	1.95	70.1	49.2	2.16
July	77.6	48.5	2.23	78.0	50.2	3.4	80.0	50.0	3.6
August	74.1	46.2	2.35	72.7	50.9	3.5	79.0	64.8	1.7
Average	73.6	45.8	3.12	74.8	49.5	2.95	76.3	54.6	2.48

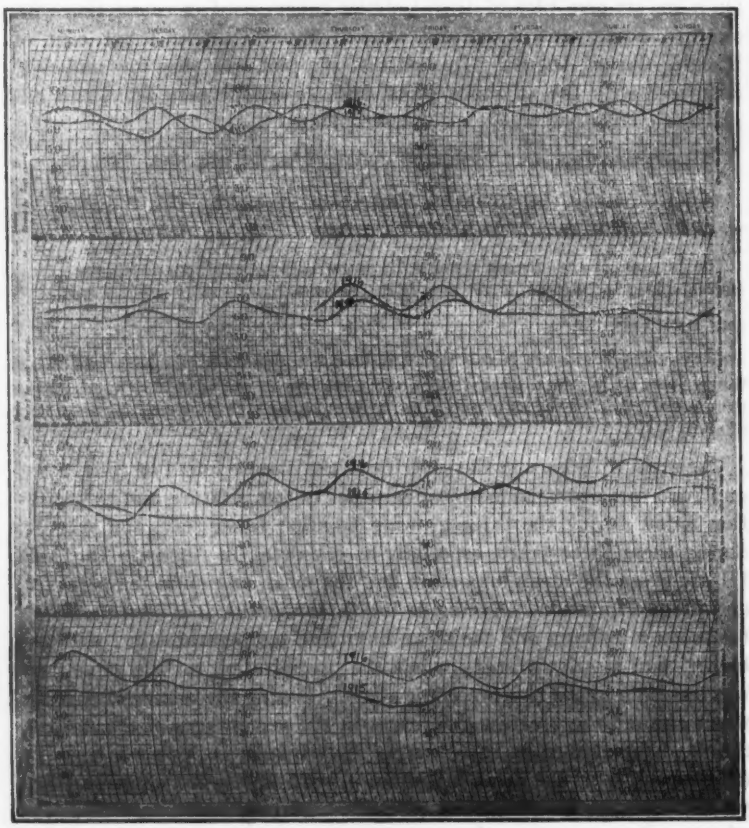

FIG. 1.—Soil-thermograph record showing the range in temperature for the month of August, 1915 and 1916 at Presque Isle, Me.

Heretofore it has been shown that there was a great difference in the air temperature during the two summers of 1915 and 1916. In figure 1 is shown a thermograph record of the soil temperature as recorded for the month of August in 1915 and 1916. These records were obtained by burying the thermograph bulb at the depth at which potatoes are planted, about 5 inches, at practically the same spot in both 1915 and 1916. An examination of this figure shows that in 1915, during the month of August, the soil temperature fluctuated between 60° and 70° and dropped below 60° twice during the latter half of the month. In 1916 the temperature was more fluctuating than for the same month the previous year and varied from 60° to 70° the early part of the month, reaching a maximum of 70° to 80° the last part of the month.

A close correlation therefore appears to exist between high temperature and little rainfall with a small percentage of blackleg, and vice versa.

SUMMARY

(1) No evidence could be obtained to indicate that the blackleg organism, under the winter conditions that existed during 1915–16 and 1916–17 in Aroostook County, Me., and during 1916–17 at Norfolk, Va., can live over in the soil or in diseased tubers that may remain there.

(2) Weather records show that the winter of 1915–16 was not an unusual one for Aroostook County.

(3) The severity of the disease during the growing season is closely correlated with temperature and precipitation and is dependent upon them. A high temperature and low precipitation tend to diminish the disease, while a low temperature and high precipitation produce conditions favorable for it.

A NEW BACTERIAL DISEASE OF GIPSY-MOTH CATERPILLARS[1]

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INTRODUCTION

During the summer of 1915 a large series of eggs of the Japanese gipsy moth (*Porthetria dispar* Linnaeus) were hatched. These eggs had been obtained for the writer by Prof. Richard Goldschmidt from Ogi, Japan. On reaching the third stage many of the caterpillars began to die of a peculiar disease which the writer had never in previous years noticed in any of his American cultures. The infection later spread to the American race, and the most rigorous methods of isolation and disinfection had to be inaugurated in order to save most of the cultures from extinction. The disease was very soon controlled, and this led at once to a belief in its bacterial origin, and its distinctness from wilt (polyhedral disease).[2] Anyone who has worked with wilt knows with what great difficulty this disease is controlled once an epidemic gains a foothold.

Inspired by the belief that this new disease might be used in combating the gipsy moth in the field, the writer made a systematic study of it during the seasons of 1915, 1916, and 1917.

SYMPTOMS AND CHARACTERISTICS OF THE DISEASE

When a caterpillar contracts this new disease, which may be provisionally named the "Japanese gipsy-moth disease," it acquires a violent form of diarrhea, loses its appetite, and finally ceases to eat. The insect seems to lose all muscular coordination and usually crawls slowly to some elevated place, where it soon dies. After death it hangs in a flaccid manner by its prolegs, with an appearance of death from wilt. In contradistinction to wilt, however, the skin does not rupture, but is so tough that one can pick up and stretch the animal with considerable force before the skin breaks.

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[1] Contribution from the Bureau of Entomology, U. S. Department of Agriculture, in cooperation with the Bussey Institution of Harvard University (Bussey Institution No. 140).

[2] GLASER, R. W. WILT OF GIPSY-MOTH CATERPILLARS. *In* Jour. Agr. Research, v. 4, no. 2, p. 101–128, pl. 11–14. May 15, 1915. Literature cited, p. 127–128.

CHAPMAN, J. W., and GLASER, R. W. FURTHER STUDIES ON WILT OF GIPSY MOTH CATERPILLARS. *In* Jour. Econ. Ent., v. 9, no. 1, p. 149–163. 1916.

GLASER, R. W., and CHAPMAN, J. W. THE NATURE OF THE POLYHEDRAL BODIES FOUND IN INSECTS, *In* Biol. Bul., v. 30, no. 5, p. 367–390. May, 1916. Bibliography, p. 383–384.

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A microscopic study of smears from the dead animals readily precludes the possibility of wilt. At times an animal will contract wilt as well as the Japanese disease, but such cases are exceptional. Therefore, when caterpillars which die from the new disease are examined, polyhedra are not found, but large numbers of a streptococcus which the writer has described under the name "*Streptococcus disparis*" are present. Sections demonstrate that this bacterium, during the early stages of the disease, is found throughout the alimentary tract. Later, and especially after death, the intestinal epithelium disintegrates and ruptures, liberating the organisms into the body cavity, where they invade practically all the tissues. Naturally species of bacteria different from *S. disparis* are also quite frequently found in the gut (intestinal flora), so that a pure culture cannot be obtained by inoculating culture tubes with material from the stomach or intestines.

During the earlier stages of the disease, when the animals contract diarrhea, the semiliquid feces everywhere soil the food plants. This fecal matter is full of the microorganism in question, and is the principal cause for the rapid spread of the infection.

The writer has isolated *S. disparis* many times from cases of the Japanese disease and has never failed to reproduce the malady. The organism was always again recovered by plating and other successful infections performed with the pure culture.

LABORATORY EXPERIMENTS

Inoculation experiments were not thought necessary, for the reason that the bacterium does not originally invade the blood of the insect. *S. disparis* invades the alimentary tract with the ingested food, and therefore food-infection experiments were considered more instructive.

Tables I to III are self-explanatory. The animals used in the experiments came from a selected stock of American caterpillars from which the wilt disease had been nearly eliminated. Gipsy-moth caterpillars taken directly from the field can not be used for experimentation, for large numbers are invariably infected with wilt. A stock of caterpillars comparatively free from this disease must first be produced by selection and this, as the writer has shown in a previous publication, takes at least three years. Each animal used in the streptococcus experiments was isolated in a separate sterile glass fruit jar and the food foliage was shipped daily from a region where the gipsy moth does not occur. This latter precaution was taken in order to preclude the introduction of wilt. The nutrient bouillon cultures were diluted one-half with sterile water and sprayed on the foliage by means of a fine atomizer until the leaves were visibly wet. Controls accompanied all of the experiments and are absolutely necessary to the proper interpretation of all disease experiments with insects.

TABLE I.—*Results of infection of eight fourth-stage American Porthetria dispar larvæ fed with red-oak leaves sprayed with a 72-hour bouillon culture of Streptococcus disparis. Bacterium isolated from alimentary tract of animal which had died from disease* [a]

Number of days	1	2	3	4	5	6	7	8	9	10	11	12	13
Number of deaths	....	....	....	....	....	....	....	2	2	....	....	....	1

[a] Three pupated and emerged. Eight controls accompanied this series. All lived, pupated, and emerged. Five dead animals tested and *S. disparis* recovered.

TABLE II.—*Results of infection of eight fourth-stage American Porthetria dispar larvæ fed with red-oak leaves sprayed with a 72-hour bouillon culture of Streptococcus disparis recovered from one of the dead animals used in previous experiments* [a]

Number of days	1	2	3	4	5	6
Number of deaths	....	1	1	1	2	1

[a] Two pupated and emerged. Eight controls accompanied this series. All lived, pupated, and emerged. Six dead animals tested and *S. disparis* recovered.

TABLE III.—*Results of infection of 25 third- and fourth-stage American Porthetria dispar larvæ fed with apple leaves sprayed with a 72-hour bouillon culture of Streptococcus disparis* [a]

Number of days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Number of deaths	....	....	....	....	....	2	....	2	1	1	9	4	3	2	....	1

[a] Twenty-five controls accompanied this series. One died of wilt; the remaining ones lived, pupated, and emerged. In the infection experiment the two which died on the sixth day succumbed to wilt (polyhedral disease); the remaining ones died of the new disease. The one which died on the ninth day, the three on the eleventh day, the two on the thirteenth day, and the one on the sixteenth day were tested and *S. disparis* recovered. The remaining dead were merely examined microscopically.

At the conclusion of each experiment it is stated that the dead animals were "tested." This signifies that stained smears were studied, the material was "plated out" on agar, and the species of bacteria isolated was observed culturally and biochemically. (See description of *S. disparis* on pages 520–521.)

From an examination of Tables I to III it will be seen that the period from infection to death varies considerably. This is probably due to individual resistance or to differences in the number of organisms ingested. Death may be expected any time after about 24 hours; indeed, it may be postponed for as long as 16 days. In Experiment I three and in Experiment II two animals lived and transformed into moths. At present no definite explanation can be offered for this occurrence. These five caterpillars became infected because the contaminated food was never removed until nearly all of it had been eaten. By confining the caterpillars in glass fruit jars having tin screw tops the food foliage can be kept palatable to the animals for three and sometimes four days.

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The writer performed three times as many infection experiments as those outlined, but since the results were similar, a repetition here would be superfluous.

In a large experiment like the one given in Table III it is hopeless to analyze thoroughly every death, on account of the enormous work involved; but it is hoped that the number carefully studied will represent a fair sample of all the deaths. Of course every dead caterpillar was examined microscopically, but by careful analysis is meant the systematic study of all species of bacteria isolated.

An attempt was made to infect 10 silkworms (*Bombyx mori* Linnaeus) and 10 army worms (*Cirphis unipuncta* Haworth) with *S. disparis*, but the organism does not seem to be pathogenic to these two insects. Guinea pigs and rabbits were forced to drink pure cultures of the organism, but failed to develop any distressing symptoms. If the disease is to be introduced into the field, it appears highly important to determine whether the organism would prove pathogenic to mammals like horses, cows, pigs, dogs, and even to human beings. After the guinea pig and rabbit experiment the writer mustered enough courage to drink 5 c. c. of a pure bouillon culture of *S. disparis*. Up to the date of writing, about seven months, he has failed to notice any distressing symptoms.

PATHOLOGY

The pathology of the Japanese gipsy-moth disease is quite interesting and distinct. Of course during the later stages of the disease practically all of the tissues are affected, but the most striking early changes occur in the muscle tissues. Plate 54, A, is a reproduction of a photomicrograph of normal and early pathological muscle tissue. The strands to the right and left are normal, whereas the muscle strand in the middle does not show the striæ so clearly, and the individual fibrillæ seem to be more loosely arranged. Plate 54, B, shows a later stage in the progress of the disease. Here the muscle tissue has lost its striated appearance, which, as can be seen, is due to the fact that the fibrillæ have lost their compactness and have separated from one another like threads of cotton. The sarcolemma disintegrates gradually with the rest, and the nuclei of the cells lose their normal positions and become scattered. Finally (Pl. 54, C) the muscle tissue disintegrates completely, the fibrillæ, etc., are no longer visible, and the whole simulates coagulated protein material with minute granules scattered throughout. When this stage in muscular disintegration has arrived, nearly all of the other tissues have likewise disintegrated more or less and *S. disparis* may now be seen scattered everywhere. All of the pathological changes cited above are much more strikingly accentuated in artificially infected animals than in those which become infected naturally. This is to be expected, for the reason

that the number of organisms ingested in artificial infections is enormous, and consequently more toxins are elaborated, which in turn cause more striking changes. The pathology of the musculature is characteristic enough, however, so that any one can diagnose the disease from sections. On finding the muscles in the conditions illustrated by figures A and B of Plate 54, one can safely predict that *S. disparis* will be found in the alimentary tract.

At the end of this article is a detailed description of *Streptococcus disparis* with an account of the media best suited to its cultivation.

Recently Paillot [1] described a microorganism parasitic in gipsy-moth caterpillars. He named the bacterium *Diplococcus lymantriae* after the old generic name of the gipsy moth, Lymantria. The writer has carefully studied Paillot's description and finds absolutely no resemblance between his organism and *S. disparis* in either cultural or biochemical characters. Moreover, *D. lymantriae* is not very pathogenic to the caterpillars, whereas *S. disparis* is highly pathogenic.

The writer has searched the literature, but has been unable to find a description of a bacterium which in any way resembles *S. disparis*. For this reason, and especially since the organism may prove to be of some economic importance, its description as a new species seems justified.

FIELD EXPERIMENTS

During the summer of 1917 Mr. A. M. Wilcox, of this Bureau, and the writer conducted a large series of field experiments in Massachusetts in connection with *S. disparis*. One important phase of the work consisted in attempting to discover whether the organism occurred anywhere in the gipsy-moth infested territory. Efforts to find the disease in 1915 and 1916 failed, and in 1917 a much more systematic endeavor to find the organism in the field, in places where it was not artificially introduced, was unsuccessful. Hundreds of dead caterpillars from a large variety of places were collected every week and brought to the laboratory for study. The mortality was always found to be due either to parasitism by the tachina fly *Compsilura concinnata* Meigen to other insect parasites, or to wilt or some disease other than the Japanese bacterial malady. *S. disparis* was recovered from none of these localities, and therefore the statement that the new disease has not occurred in this country in the field prior to 1917 seems justified.

Another phase of the work consisted in introducing the disease by a variety of methods in woods heavily infested by the gipsy moth. The idea of the writer is not to attempt the extermination of the gipsy moth with the new bacterium, although, of course, he would wish to do so, if it were possible, but to approach the subject in the same spirit in which

[1] PAILLOT, A. MICROBES NOUVEAUX PARASITES DES CHENILLES DE LYMANTRIA DISPAR. *In* Compt. Rend. Acad. Sci. [Paris], t. 164, no. 13, p. 525-527. 1917.

the Bureau of Entomology of the United States Department of Agriculture has approached all of the other parasite-introduction work. The attempt is merely being made to introduce another foreign parasite which, if it becomes established and becomes as effective in the woods as it is in the laboratory, will prove highly beneficial. It should be added that when an epidemic of the new disease breaks out in the laboratory the percentage of mortality far surpasses the percentage of mortality in a laboratory epidemic of wilt. Every one familiar with the subject knows that wilt has been a very important factor in saving the New England forests from utter ruin. If, therefore, the matter is approached in the spirit of "parasite introduction," the economic use of insect diseases assumes a new light and should prove extremely fruitful in combating many of our noxious pests.

Contrary to expectations, the field experiments have proved highly successful in so far as the introduction and recovery of *S. disparis* is concerned. In other words, the disease has been reproduced in the field. Indeed, in two localities in Massachusetts—namely, Sherborn and North Carver—it was possible to produce quite a severe epidemic. Notwithstanding the encouraging results, however, no statistics will be given until another season has passed. A large amount of work is still needed to determine the relative importance of this method of combating the gipsy moth.

Streptococcus disparis, n. sp.

MORPHOLOGY.—From 1.5 per cent neutral nutrient agar. From 1.5 per cent neutral potato agar. From milk. From neutral nutrient bouillon.

Organism examined in these media after 24, 48, and 72 hours, after one week, and each month for eight months. No decided variations in morphology observed. Division occurred in one direction of space. Chains of 3 to 4 units frequently seen in liquid media. Diameter not 1 M. Capsulated. Motility O. Gram-positive. Stains readily. Typically a streptococcus.

NUTRIENT AGAR STROKE, 1.5 per cent.—Neutral. Growth in five days at 35° C. scanty, beaded, flat, glistening, smooth, white, opaque, odor absent, butyrous, medium unchanged.

POTATO AGAR STROKE, 1.5 per cent.—Neutral. Growth in five days at 35° C. abundant, spreading, flat, glistening, smooth, white, opaque, odor absent, butyrous, medium unchanged.

POTATO.—Growth moderate, spreading, flat, odor absent, butyrous, color of medium unchanged.

GELATIN STAB.—Growth best at top. Line of puncture beaded. No liquefaction. Medium unchanged.

NUTRIENT BROTH.—No ring, no pellicle, clouding slight, clearing after 15 days, slight sediment, odor absent.

MILK.—Coagulation delayed. Extrusion of whey. Color of medium unchanged. No peptonization.

LITMUS MILK.—Acid, prompt reduction, coagulation delayed, extrusion of whey, no peptonization.

DUNHAM'S PEPTONE SOLUTION.—Clouding very slight. Growth poor.

GELATIN COLONIES.—Growth slow, colonies very small and majority under surface. Surface colonies round, slightly convex, edge entire, no liquefaction.

NUTRIENT AGAR COLONIES.—Growth slow. Majority of colonies under surface and oblong. Surface colonies round, smooth, convex, edge entire, internal structure finely granular. Diameter 0.25 to 0.33 mm.

POTATO AGAR COLONIES.—Growth rapid. Majority of colonies under surface and oblong. Surface colonies round, smooth, convex, edge entire, internal structure finely granular. Diameter 1 to 1.5 mm.

NH_3 PRODUCTION.—Absent.

NITRATE SOLUTION.—Nitrates not reduced.

INDOL PRODUCTION.—Absent.

HYDROGEN SULPHID PRODUCTION.—Absent.

FERMENTATION OF CARBOHYDRATES WITH FORMATION OF ACID AND GAS.—

	Gas.	Acid.
Dextrose	o	+
Levulose	o	+
Saccharose	o	+
Maltose	o	+
Lactose	o	+
Mannit	o	+
Adonit	o	+
Dulcit	o	+

OXYGEN REQUIREMENTS.—Facultative anerobe.

BEST MEDIA FOR CULTIVATION.—Solid: 1.5 per cent, neutral potato agar. Liquid: Neutral nutrient bouillon containing a carbohydrate, especially bouillon containing about 1 per cent of saccharose, maltose, or mannit.

PATHOGENICITY.—Pathogenic to the caterpillars of the American, European, and Japanese races of the gipsy moth (*Porthetria dispar* Linnaeus). Not pathogenic to silkworms (*Bombyx mori* Linnaeus) and army worms (*Cirphis unipuncta* Haworth) when fed *per os*. Guinea pigs, rabbits, and human beings when fed pure culture *per os* not affected.

SUMMARY

(1) In 1915 a new infectious disease was found in certain cultures of the Japanese race of the gipsy moth.

(2) The infection spread later to cultures of the American race.

(3) The disease is clinically, pathologically, and etiologically distinct from wilt.

(4) A streptococcus was found to be the causative agent.

(5) The bacterium is new to science and is here described under the name "*Streptococcus disparis*."

(6) During the early stages of the disease the bacterium is found throughout the alimentary tract of the gipsy-moth caterpillars.

(7) During the later stages of the disease and after death the bacterium invades practically all the tissues.

(8) *S. disparis* invades the alimentary tract with the ingested food.

(9) Healthy animals naturally become infected by eating food soiled by the feces of infected animals.

(10) *S. disparis* is not pathogenic to silkworms (*Bombyx mori* Linnaeus) or to army worms (*Cirphis unipuncta* Haworth).

(11) *S. disparis* is not pathogenic to human beings, guinea pigs, or rabbits.

(12) The most striking pathological changes during the course of the disease occur in the muscle tissues of the caterpillar.

(13) Many observations and tests show that the new disease did not occur in this country prior to 1917.

(14) Field experiments were conducted with *S. disparis* in sections of the gipsy-moth infested territory.

(15) Success was obtained many times in reproducing the disease in the field.

(16) In two places quite a severe epidemic was created.

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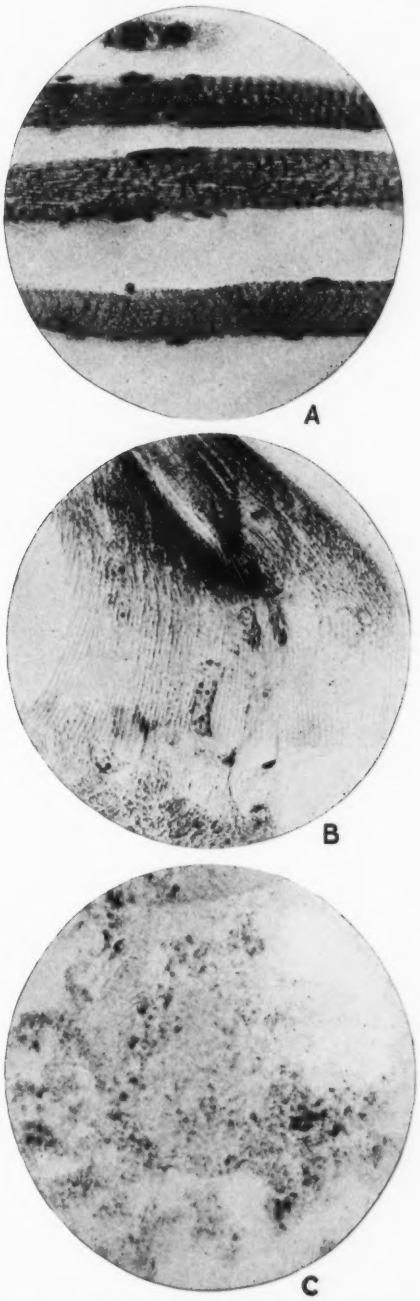
PLATE 54

A.—Photomicrograph of normal and early pathological gipsy-moth muscle tissue. × 540.

B.—Photomicrograph of late pathological gipsy-moth muscle tissue showing separation of fibrillæ. × 540.

C.—Photomicrograph of last stage in pathology of gipsy-moth muscle tissue, showing complete disintegration. × 540.


A
B
C